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U. S. ARMY MEDICAL UNIT

FORT DETRICK, FREDERICK, MARYLAND 21701

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SPECIAL REPORT

TO THE

COMMISSION ON EPIDEMIOLOGICAL SURVEY

OF THE

ARMED FORCES EPIDEMIOLOGICAL BOARD

SYMPOSIUM ON

INFECTION AND METABOLISM

Walter Reed Army Institute of Research
Washington, D. C.

September 1967

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U. S. ARMY MEDICAL UNIT
FORT DETRICK, FREDERICK, MARYLAND 21701

SPECIAL REPORT
TO THE
COMMISSION ON EPIDEMIOLOGICAL SURVEY
OF THE
ARMED FORCES EPIDEMIOLOGICAL BOARD

SYMPOSIUM ON
INFECTION AND METABOLISM

COMPILED BY
COLONEL WILLIAM R. BEISEL, MC
and
PHEBE W. SUMMERS

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FOREWORD

This is a Special Report of presentations made at the Symposium on Infection and Metabolism held 7 and 8 September 1967 at Walter Reed Army Institute of Research, Washington, D. C. and sponsored by the Commission on Epidemiological Survey. This Special Report is prepared in lieu of a formal Annual Report.

In conducting research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.



WILLIAM R. BEISEL
Colonel, MC
Acting Commander

5 February 1968

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THE DIRECTOR'S SUMMARY REPORT

The Annual Meeting of the Commission on Epidemiological Survey was held at the Walter Reed Army Institute of Research on 7 and 8 September, 1967. The two day meeting was devoted to a Symposium on Infection and Metabolism which was attended by Commission members, representatives of the Military Services, distinguished scientific contributors to the program and guests:

U. S. Army Medical Unit

Colonel William R. Beisel, MC
Captain Martha K. Ward, USPHS
Lt Colonel Stewart McConnell, VC
Lt Colonel Peter J. Bartelloni, MC
Major George E. Shambaugh, III, MC
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Commander Charles N. Miller, MC, Bureau Medicine and Surgery
Ensign Robert P. Nalewak, MSC, Fort Detrick

U. S. Air Force

Captain Amos R. Townsend, Office of The Surgeon General

Guests

Dr. Hilton B. Levy, National Institutes of Health
Dr. Elisha Atkins, Yale University
Dr. Paul M. Newberne, Massachusetts Institute of Technology
Dr. Robert L. Squibb, Rutgers University
Dr. Vernon R. Young, Massachusetts Institute of Technology
Dr. Elliot M. Levine, Albert Einstein College
Dr. John M. Woodward, University of Tennessee
Dr. Paul C. Zamecnik, Harvard University
Dr. Peter F. Bonventre, University of Cincinnati
Dr. Morton I. Rapoport, University of Maryland
Dr. Irving Gray, Georgetown University
Dr. Adam J. Rapalski, National Research Council
Dr. Hamish N. Munro, Massachusetts Institute of Technology
Dr. Sidney H. Ingbar, Thorndike Memorial Laboratory
Dr. Herbert L. DuPont, University of Maryland
Dr. Harold N. Glassman, Fort Detrick
Dr. Joseph E. Johnson, University of Florida
Dr. Bernard du Buy, University of Maryland
Dr. N. V. Barzauskos, University of Maryland
Dr. Frank A. Carozza, Jr., University of Maryland
Dr. Walter W. Kemmener, National Air Space Administration
Dr. Samuel Bessman, University of Maryland

Unavoidable conflicts prevented Dr. Gustave J. Dammin, President of the Armed Forces Epidemiological Board, from attending; he was ably represented by Dr. John S. Craighead. Captain Sidney A. Britten, USN, Executive Secretary of the Board, and Administrative Assistant, Miss Betty Gilbert, were cited for their unstinting assistance which makes it possible to conduct the Commission's affairs.

After introductory remarks by Colonel William R. Beisel and the Director, the two days were devoted to the scientific agenda and discussions. Colonel Dan Crozier was particularly cited for his foresight and attention to administrative details which made the Symposium possible. Because of illness he was unable to attend the meeting.

The U. S. Army Medical Unit, working in collaboration with scientists at Fort Detrick during the year, made significant contributions pertinent to better understanding of the pathogenesis of certain specific infectious diseases and provided leads to earlier etiologic detection.

AMINO ACID AND ENZYME ALTERATIONS

Among these studies is the demonstration that whole blood amino acids show circadian periodicity with values lowest at 0400 hours and highest between 1200 and 2000 hours. In controlled human infections, such as typhoid fever, blood amino acid values are lower during the incubation

period of those persons who subsequently become clinically ill; concentrations increase during the active disease. Although there are no distinctive diagnostic patterns, there are valuable leads involving single and total amino acid changes in typhoid fever, Venezuelan equine encephalitis and after vaccination with 17-D yellow fever vaccine. Related biochemical studies showed that induced pneumococcal infection in mice produced alterations in liver protein anabolism, specifically, tryptophan pyrrolase, a specific liver enzyme. Changes in enzyme synthesis and total hepatic protein anabolism occur very early in infection and such biochemical reactions presumably precede the onset of illness. Urinary diazo amines are excreted during certain infections, such as typhoid fever. Diazo amine formation is related to tryptophan metabolism through niacin pathways. Urinary diazo reactants were demonstrated early in the course of human sandfly fever. Interpretation of enzymatic abnormalities are fraught with difficulties since techniques require standardization; valuable leads are being developed.

CELLULAR NUCLEIC ACID CHANGES

During Diplococcus pneumoniae infection of mice, increased synthesis of RNA occurred 24 hr postinoculation with values below control levels in the agonal stages. In virulent arbovirus infections of mice depression of RNA synthesis occurs. The relationship of these findings to pathogenesis is unknown. Interpretation of studies of increase or decrease in RNA synthesis requires evaluation of whether cells are in a resting or growing phase. Double stranded RNA has the capacity to increase the rate of interferon in cells which increases host resistance to virus infections.

DETECTION OF EARLY ANTIBODY, ANTIGEN AND GAMMA GLOBULINS

Using the Ferne antibody plaque technique and identification of RNA in cells with acridine orange and other stains, it has been shown that cells begin to form antibody about 5 hr after the antigenic stimulus. Studies directed to early identification of circulatory antigen show promise. Latex particles sensitized with pneumococcus antisera show good specificity particularly when particles are incubated at 4 C. This reaction antedates the demonstration of bacteremia.

Studies have been initiated to determine the patterns of immune globulin response in volunteers given $10^{7.5}$ mouse LD₅₀ doses of 17-D yellow fever virus as the primary exposure. Titers of IgM, IgG, IgA are being evaluated.

HORMONAL RESPONSES IN INFECTIONS

Metabolic responses are altered in specific infections. Unbound thyroxine increases with infection before onset of fever in tularemia and returns to normal before defervescence. Thyroxine disposal begins several days after onset of clinical illness and PBI rises in the early recovery period. These and other results suggest that thyroxine exerts an anabolic effect during infection. In mice infected with pneumococci, plasma cortisol levels increase about 4 hr before those of plasma thyroxine.

A rather stereotyped pattern of adrenal response occurs in certain acute infectious illnesses. Glucocorticoid hormone output increases before or simultaneously with onset of symptoms; plasma 17-OCHS concentrations lose their afternoon diurnal fall and remain slightly above early morning levels. Mild infections fail to alter adrenal response; severe and protracted infections are associated with depressed adrenocortical output. Studies of protein synthesis and enzyme induction in animal infections suggest that endogenous glucocorticoids benefit the host by stimulating increased protein anabolism within the liver.

TRACE METALS AND SERUM PROTEIN CHANGES

Evaluation of trace metal changes have shown depression of urinary Zn output at the onset of human tularemia and Q fever and subsequent excess excretion in the postfebrile period. Other clues of early change are that serum glycoglobulins appear earlier in infection and are more specific than routine serum proteins. Alpha glycoglobulins are decreased in mice and rats within 12-16 hr after pneumococcal infection. Contrariwise, they are increased in humans prior to and simultaneous with the onset of tularemia. Conceivably, patterns may be identified as specific indicators of etiology in the early stages of infection.

LOCALIZATION OF STAPHYLOCOCCAL ENTEROTOXIN

Staphylococcal enterotoxin B (SEB) injected intravenously in rats and monkeys is rapidly removed by renal clearance. Fluorescent labeled and unlabeled toxin localizes in proximal renal convoluted tubules; smaller amounts appear in the liver and gastrointestinal tract. Toxin may gain access to the tubules via glomerular filtration and tubular reabsorption.

The Commission, through the University of Maryland Contract, has extended the studies of vaccines and pathogenesis, including physiologic effects, of toxins.

Q FEVER VACCINE EVALUATION

The influence of phase variation in Q fever vaccines has continued. Thirty-five hundred GPIFD₅₀ of Coxiella burnetii aerosolized as a "static cloud" will cause disease in volunteers with an incubation period of about 10-12 days. Phase I vaccine gave more protection to exposed volunteers than did Phase II. Of 19 volunteers challenged 5-8 months after Phase I vaccination, 2 developed disease. Two of 5 volunteers who had received Phase II vaccine became ill. One Phase I vaccinee was challenged 36 months after receiving a single 30 mg dose of the vaccine. He developed a low grade persistent fever requiring tetracycline therapy.

Three Rocky Mountain spotted fever convalescents developed unmodified Q fever after exposure to C. burnetii.

There have been 4 separate trials with viable Q fever. Illness was produced in 11 of 14 control subjects. In each of 3 protocols, one control failed to develop any evidence for infection. Reasons for these missed infections are unknown. Studies are directed to the identification of either specific or nonspecific inhibitory protein substances in the upper respiratory tract which might be related to humoral defense mechanisms.

ROCKY MOUNTAIN SPOTTED FEVER VACCINE EVALUATION

The study of this disease was initiated in 1967 to evaluate vaccine effectiveness. All of 13 volunteers inoculated intradermally with 10 GPIID₅₀ doses of the Sheila Smith Strain of Rickettsia rickettsiae developed typical Rocky Mountain spotted fever. Incubation periods averaged 5½ days with a range of 4-9 days. Onset of illness was abrupt with the appearance of fever, headache and myalgia. Rash occurred on the second or third day of fever; its appearance was not inhibited by antibiotics. Chloramphenicol treatment was initiated after 24-36 hr of temperature elevation over 103 F. Therapy with this drug or tetracycline was continued for 5 days. Fever usually abated in 2.5 days after beginning therapy. Five relapses occurred in 3 volunteers treated with chloramphenicol and 2 with tetracycline. The relapse phases were mild and response was prompt on reinstitution of antibiotic therapy.

Studies of rickettsemia are incomplete. Neurologic evaluations have been conducted in the Clinical Study Center of the University of Maryland Hospital. Serial electroencephalograms were normal. One of the 3 patients showed 10 lymphocytes in the spinal fluid at the peak of illness; this cleared promptly.

Two vaccinated volunteers developed illness after the infectious challenge; one had received monovalent Rocky Mountain spotted fever vaccine and the other a composite vaccine including 2 other rickettsial antigens. Studies are in progress and will include various immunizing schedules and a smaller infectious challenge. Complete serologic responses will be determined.

EVALUATION OF ORAL TYPHOID FEVER VACCINES

An oral typhoid vaccine, "Typhoral," which contains 3×10^9 organisms each of typhoid (Ty58), paratyphoid A and paratyphoid B was administered to volunteers in the dosage of 3 tablets/day on 3 successive days. This vaccine has been used by the German Army since 1960. No systemic reactions were noted in 103 volunteers vaccinated in February and March, 1967. One hundred of the volunteers showed the following antibody titers: Somatic (O) 11%, H 3%, and Vi 16%. The titer rises were 4-fold in 60% of the group.

A monovalent Swiss vaccine, "Taboral," containing Salmonella typhi Ty2 (100×10^9 organisms) was given to 88 volunteers in July and December, 1967, in doses of one tablet twice daily for 3 days. No systemic reactions

occurred. Antibody titer responses in 25 subjects were: O antibody 4%, H, 8%, and Vi, 12%. Analysis of the remaining subjects is incomplete.

Eleven volunteers who received "Taboral" vaccine were given 100,000 viable S. typhi (ID₅₀ dose) orally of the Quailes strain in November, 1967. Four of 11, or 36%, developed disease. One of the 4 relapsed and required additional antibiotic therapy; this volunteer had shown Vi antibody following vaccination. The attack rate in volunteers was 38%, which indicates that the killed oral vaccines used are not as effective as the killed vaccines given parenterally.

SIMULTANEOUS INFECTION

The response to simultaneously administered living vaccine strain (LVS) tularemia and Q fever has been studied. The LVS strain of tularemia given by aerosol in large doses, after a 3-day incubation period, produces a mild, self-limiting febrile illness and a prompt serological response. Thirty-five hundred GPIFD₅₀ doses of Q fever rickettsiae is the standardized challenge dose for vaccinated volunteers. Illness in control subjects begins in about 11 days. Several clinical patterns emerged when these organisms were simultaneously aerosolized and inhaled by 17 volunteers. In 7 there was definite synergism: Q fever appeared much earlier than expected. The initial fever caused by LVS did not abate completely. It appeared to blend into a continuous pattern thought due to C. burnetii since there was no response to streptomycin therapy. There was a rapid defervescence following institution of tetracycline. Two separate illnesses were observed in 9 volunteers. One volunteer was given streptomycin with the onset of fever; a second illness did not occur. Serological studies are incomplete. These should clarify the presence of antigenic interference or competition.

STUDIES OF ENDOTOXIN TOLERANCE

Studies of the mechanisms of the acquisition of human endotoxin tolerance were originally designed to evaluate the role of endotoxemia in the pathogenesis of the febrile and toxic course of Gram-negative bacterial infections. (Findings reported previously to the Commission; latest report documented in the Transactions of the Association of American Physicians, 1967; in press.) Focus is now directed to developing methods to combat the effect of overwhelming endotoxemia, i.e., active or passive means of protection. Such studies require further clarification of the mechanisms of endotoxin tolerance.

The importance of antibody in tolerance to endotoxemia is unknown. Quantitative measurements of the febrile and antibody responses of splenectomized rabbits and man to repeated intravenous injections of bacterial endotoxins show that the splenectomized host is no more responsive to the initial injection of endotoxin, yet it develops active and passively transferrable tolerance significantly more slowly than do intact

control subjects. Additional studies were performed in partially hepatectomized rabbits to determine whether such retarded acquisition of tolerance in the splenectomized is based upon decrease in total reticuloendothelial mass or to lack of this major immunologically competent tissue. Despite ablation of a more functional reticuloendothelial system (determined by uptake of Au¹⁹⁸) than in the splenectomized host, no retardation of tolerance was observed. These findings suggest that antibodies do mediate tolerance, and work is currently in progress to characterize these antibodies and quantitate their efficacy during experimental endotoxemia.

TOLERANCE TO STAPHYLOCOCCAL ENDOTOXIN

Studies initiated at Fort Detrick and continued at the University of Maryland show that SEB is highly pyrogenic for rabbits; fever is probably mediated through the release of endogenous pyrogen. Rabbits vary greatly in their pyrogenic response to an initial intravenous SEB challenge but a dose response relationship can be established. As few as 3 single daily intravenous injections induce a transient pyrogenic refractory state probably mediated by specific sensitization. Repeated intravenous enterotoxin challenge over a period of several months induces a more lasting pyrogenic tolerance probably due to protective serum antibody. Subsequent studies of pathogenesis will attempt to correlate initial pyrogenic sensitivity to enterotoxin and the infectious reaction to the parent staphylococcal strain as well as the effect of enterotoxin desensitization on such infections.

VASCULAR EFFECTS OF CHOLERA TOXIN

A toxic fraction of Vibrio cholera, designated as Craig's permeability factor, has been shown to provoke dilatation of arterioles and sluggish reaction to epinephrine in the rat meso-appendix after oral administration. Further studies of the effect of cholera toxin in the micro-circulation are in progress.

Theodore E. Woodward, M.D.
Director
Commission on Epidemiological Survey

INTRODUCTION
to the
SYMPOSIUM ON INFECTION AND METABOLISM

Colonel William R. Beisel, MC*

For a period of several years the U. S. Army Medical Unit has looked forward to a meeting that would bring together a key group of investigators for wide-ranging discussions concerning metabolic aspects of infectious illness.

This paper will serve as both an introduction and as a keynote presentation for the subject matter which follows. Important facets of the mission of the Medical Unit include: (1) the search for new and improved methods for the rapid diagnosis of infectious illness and (2) the development of methods for the prevention, suppression, and/or treatment of infectious illness. Each of these individual aspects of the mission demands an improved understanding of the mechanisms a normal host employs to resist infection. To achieve these goals a variety of investigative techniques has been used to study metabolic changes within the host.

It is hoped that this session of the Commission on Epidemiological Survey will serve to review and evaluate critically the results of this direction of research effort which is now in its fifth year. To this end, it has proved possible to develop the agenda for this year's meeting, in the format of a "Working Symposium." This meeting enables us to bring together for the first time, a large number of individuals whose primary research efforts are directed toward studies of host responses to infection, along with other invited guests whose opinions are sought because of the depth of their knowledge in closely related fields. This then is a unique group of individuals whose collective expertise encompasses a variety of investigative disciplines. It is hoped that the program will provide a platform for open discussion and exchange of ideas and concepts, and thereby pave the way for future efforts.

The application of metabolic techniques for study of the infected human host is not a new one. Prior to the turn of the century, as methods became available to measure various elements and compounds in biological fluids, studies were initiated in patients suffering from a variety of acute infectious diseases. The catabolic effects of infectious illness on nitrogen balance were demonstrated quite early; they were soon shown to be minimized by increasing the caloric content of the diet while insuring an adequate intake of protein. Changes in

* U. S. Army Medical Unit.

oxygen consumption, respiratory quotients, and chemical constituents of the blood were all reported, including the generalized tendency for serum electrolytes to fall during various types of infection despite a markedly diminished excretion of Cl and Na in the urine. Such studies in human subjects were published frequently for a number of years, but then all but disappeared from the literature following the introduction of specific antibiotic drugs into clinical medicine.

In the decade following World War II, several new approaches and methods of metabolic study were introduced to investigations on infectious disease. Nutritional aspects of host susceptibility and resistance were emphasized as fields for fruitful study by the work and reviews of Scrimshaw and his co-workers.^{1,2/} Research concerning alterations in metabolic functions of various biochemical pathways in experimentally infected animals began to appear. However, until recently, publications of such a nature were initiated only by a small number of groups; of these, publications by L. J. Berry's team concerning intermediary carbohydrate metabolism^{3/} and host enzyme synthesis,^{4/} John Woodward's work concerning amino acid metabolism in experimental tularemia,^{5-8/} Eric Reiss's pioneer studies of isotopic nitrogen kinetics during streptococcal infection,^{9,10/} Robert Squibb's investigations involving hepatic and muscle nucleic acid, protein, and amino acid interrelationships,^{11,12/} Paretsky's work with Q fever,^{13,14/} and Cora Downs' publications^{15-17/} concerning the metabolic consequences of overwhelming infection all impress me as being important milestones that have set the stage for more current investigations.

Running in parallel with these efforts have been the more basic studies conducted in numerous laboratories in which the infected host under scrutiny was a bacteria and the infecting microorganism was a bacteriophage. Advances in techniques for maintaining tissue cells alive in cultures have permitted similar studies of infection within single individual mammalian cells. Other new methods make it possible to pinpoint changes to subcellular fractions or organelles of cultured cells or whole organ homogenates. It is now becoming possible to interpret such investigations in terms of the entire animal or human host.

Our own metabolic studies within the Medical Unit and the recent efforts of a number of other investigators here today, have centered about an important aspect of the infectious process that heretofore has generally been neglected, that is, the period of incubating illness. We began our studies with an attempt to define the broad patterns of metabolic change in the infected human host; to accomplish this goal, we employed carefully controlled, prospective investigations of metabolic balances of a number of elements.^{18-22/} The results of these studies are published elsewhere.^{18-22/} Such investigations served to establish a number of fundamental points as shown diagrammatically in Figure 1.

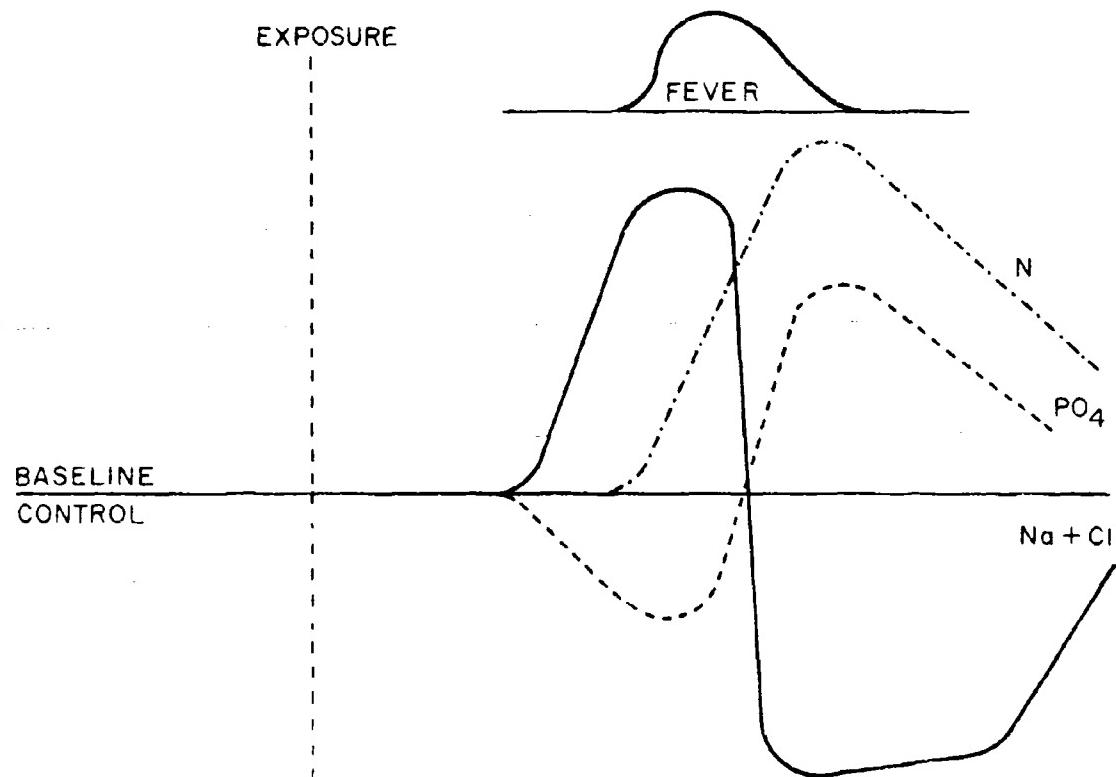


FIGURE I. URINARY LOSSES IN FEBRILE INFECTIONS.

The first of these, was the observation that very little in the way of a broad or generalized metabolic change occurred until the onset of clinical illness or immediately before the onset of illness. While more recent studies indicated that early incubation period changes of a biochemical nature could be found, if sought by appropriate measures, the subtle nature of these changes failed to be reflected in most of the balance studies.

The major metabolic response to acute infectious illness became evident as a catabolic process that was generalized in nature and remarkably stereotyped in pattern, despite its complexity. This schematic pattern held true for each of the 3 types of diseases studied: bacterial tularemia, rickettsial Q fever, and viral sandfly fever. The only prominent metabolic differences among these illnesses were in the length of their incubation periods. Significant metabolic events could all be related in times of onset to the beginnings of fever. There were increasing losses of the major intracellular elements as shown by the N curve. These losses began in coincidence with symptomatic illness and did not reach a peak until after the onset of clinical recovery. The major intracellular elements, including N, Mg, K, and P were all lost in proportional amounts. Of these, P initially followed a slightly different pattern, in that just prior to and during the onset of fever there was a consistent decrease in its loss in urine. Evidence has been presented previously that this transient renal retention of P may be related to hyperventilation and respiratory alkalosis during mounting fever.^{20/} An increased loss of Na and Cl from the body was also a consistent pre-febrile and early febrile event that was followed in turn by renal retention of these electrolytes coincident with an increase in aldosterone secretion.^{19/} This overall pattern of events, in all its complexity, constituted the catabolic process in each of the diseases studied.

The duration of the catabolic phase was long, and the cumulative losses engendered during catabolism were not regained for a period of several weeks following clinical recovery. The magnitude of the catabolic phase could be related to both the duration and severity of illness.

Negative balances during infectious illness arose from a combination of causes: diminished dietary intake, increased losses through the urine, increased losses through the skin associated with febrile sweats, and increased losses from the gastrointestinal tract associated with vomiting or diarrhea.

In other similar balance studies in noninfected subjects, evidence was obtained indicating that impaired dietary intake and the presence of fever, *per se*, were the major factors leading to catabolic losses. In contrast, it was found that the measured adrenal glucocorticoid response contributed little if anything to the catabolic events during acute infectious illness.

In addition to providing an understanding of the catabolic patterns of response to acute infection, the prospective nature of the early balance studies did permit the recognition in several instances of a subtle but highly important finding: in patients who experienced only mild illness after their exposure, or in patients who were protected from illness because of active immunity, there was noted frequently a small but definite period of N retention that followed soon after an exposure to infectious microorganisms. This period of early retention was interpreted by us to represent an anabolic response involving protein synthesis early after the entry of invading organisms into the host.

Other evidence, well known to all of you, indicates that definite anabolic events do accompany acute infection. A number of these are listed in Table I. All involve the synthesis of new protein.

TABLE I. ANABOLIC RESPONSES TO INFECTION

Leukocytosis
Tissue enzyme induction
α -globulins
Interferon
Antibody proteins
Nucleic acid synthesis
Positive nitrogen balance

In considering the evidence supporting the existence of infection-stimulated anabolism as well as infection-stimulated catabolism, it seems probable that the simultaneous occurrence of both directions of change would result in an algebraic summation of these effects when considered from the point of view typical of body balance studies. In contrast to catabolism as a detrimental effect, it seemed attractive to postulate that anabolic events represented by attempts by an intact host to defend its integrity against infectious invaders, and further, that such attempts might represent primary host responses of a metabolic nature. This concept lead to many of the studies which follow.

Two years ago, in a Presidential Address delivered to the clinical investigators assembled in Atlantic City, Dr. Barry Wood called attention to the growing complexity of knowledge developed by the study of molecular biology. He pointed out, in clear terms, the necessity for continuing attempts to bridge and interrelate the newer concepts of cellular function with the older, established approaches to basic and clinical investigation as well as to the bedside teaching and practice of medicine. In a sense, this Working Symposium represents such an attempt at establishing meaningful correlations. Hopefully, it will be found that work presented throughout this symposium will also reveal an attempt by various individuals to design their own studies to provide such bridges between molecular changes and whole animal responses.

In introducing this subject matter, I admit to considerable bias in favor of continued metabolic studies as a basis for both theoretic and practical advances. It has not yet been possible to achieve the ultimate goal of diagnosing with consistent accuracy the presence, early in the incubation period, of an impending generalized infectious illness. However, by means of a relatively simple procedure it is possible at the present time to predict with an excellent degree of accuracy those individuals within a group of volunteers exposed to virulent tularemia or typhoid organisms, who will develop symptomatic illness a day or so later. A specific etiologic diagnosis cannot be made on the basis of metabolic changes; there are preliminary indications, however, that by use of a rapid analysis of serum glycoproteins, it may be possible to differentiate bacterial from viral illness during the first day of symptoms. Dr. Klainer's discussion appears later in this report.

From a therapeutic approach, descriptive studies of a metabolic nature provided a rational basis for fluid and electrolyte replacement therapy in Asiatic cholera, an approach to treatment that has proved highly efficacious. Our balance studies reemphasize the need for an increased intake of calories, minerals, and protein to minimize the catabolic effects of acute infection. A somewhat different approach, based on the molecular pathophysiology of intracellular virus proliferation, may eventually make it possible to prevent or treat viral infection by the employment of atypical nucleotides as drugs. Dr. Zamecnik will discuss his work along these lines. Apart from this meeting, but similar in concept, has been the publicity appearing on financial pages of newspapers throughout the country to the effect that extracts of RNA obtained from microorganisms may serve to induce interferon production by host cells, and thereby to provide a short-term protection against viral infections.

Investigations of this nature presented here involve both careful descriptions of events not previously known to be associated with acute infectious illness along with studies of possible physiologic, metabolic, biochemical, and molecular mechanisms that might logically account for described changes. It has long been obvious that the entry of an infectious organism into an intact host initiates a highly complex set of interactions within the host. These have by custom been subdivided into responses categorized as either specific or non-specific. There is some question about the present real value of such a division; such nonspecific factors as the hormonal responses, fever, nutritional status, general toxicity and the like, certainly wield an influence on all other host responses. Attempts to differentiate primary from secondary responses of the host are likewise difficult to define, and we have hardly begun to face up to potential differences in mechanism between host responses to generalized infections in contrast to infections that remain localized. Still less obvious are the metabolic aspects of latent viral infections that may flare up to produce recurrent herpetic lesions or canker sores, or to the existence

of latent infections with incomplete viruses that cannot propagate until the host cell is invaded by a so-called "helper" virus. Attempts to define such infections in terms of specific host metabolic responses seem well beyond our current capabilities or the scope of this meeting. On the other hand, it is reasonably sure that information presented during this 2 day session will provide new and exciting concepts concerning the nature and extent of host responses that can be studied with currently available techniques. Many of these studies are quite preliminary in nature at this time, but it is hoped that they may serve as a basis for future advances.

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SECTION I

AMINO ACID AND ENZYME ALTERATIONS IN THE HOST

MODERATOR: Dr. Theodore E. Woodward

DISCUSSANT: Dr. Samuel Bessman

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11

INTRODUCTION TO SECTION I

Theodore E. Woodward, M.D.*

Colonel Beisel keynoted this symposium very nicely. His provocative comments stress the need for proper understanding of mechanisms of disease, i.e., pathogenesis. For many years we have depended almost entirely on antibiotics for therapy of infectious diseases. Antibiotics are extremely useful and act by interfering with microbial reproduction in various ways. Yet they have not solved all of our problems. Patients still die of infections even when caused by antibiotic-sensitive microorganisms. We understand too little regarding the influence of infectious agents or their toxins on cellular metabolism, the vascular system and the ability of the host to resist infection.

Colonel Beisel cited Asiatic cholera as an example of a microbial disease that could be cured in humans without administering an antimicrobial agent. The therapist needs only to replace proper amounts of body fluids and electrolytes which have been lost via the intestine. Clinical investigators at the SEATO Research Cholera Laboratory in Dacca have treated 400 patients successfully using properly applied nonspecific measures. These studies were directed by Doctors Abram S. Benenson and Robert Phillips.

Colonel Beisel and his staff hope to gain knowledge of the proper key pieces of information about other infectious diseases. Eventually it might be possible to treat by replacing some essential metabolite, substrate or cellular ingredient that has been taken from the host cell, destroyed or made inert, by a microbe or its toxin. Such information will place therapy on a fundamental rather than an empiric basis. Good progress is being made in these directions and possibly the results of investigations presented at this meeting will bring us closer to this goal.

* University of Maryland School of Medicine, Baltimore, Maryland.

WHOLE BLOOD AMINO ACIDS IN INFECTIOUS DISEASES

Captain Albert S. Klainer, MC*

From studies reported previously at these meetings and from those which have continued in our laboratory over the past year, it appears that blood amino acid changes are one of the earliest and most consistently demonstrable biochemical indications of infection to occur before the onset of clinical illness.

Whole blood amino acids demonstrate a circadian periodicity characterized by values lowest at 0400 hours and highest between 1200 and 2000 hours.

Dr. Squibb^{1/} has demonstrated a circadian periodicity for serum amino acids in growing chickens. We have recently described a circadian periodicity of whole blood amino acids in men and mice.^{2/}

Figure 1 shows this periodicity averaged for 6 normal subjects over a 5-day period. The shaded areas represent periods of sleep. The figures on the left represent the total integrated value for 0.006 ml of whole blood; this is an absolute term representing total amino acid concentration as determined densitometrically. The concentration of amino acids on any given day may be different from that obtained on any other day but the rhythmicity observed is the same from day to day. While the concentration of amino acids in any single individual may be different from that of any other individual even though diet and environment are the same, the periodicity is similar.

At these meetings a year ago Dr. Feigin described amino acid changes in respiratory acquired tularemia and following immunization with live attenuated Venezuelan equine encephalitis (VEE) virus vaccine.^{3/} Tularemia was characterized by a significant fall in total blood amino acid preceding the onset of clinical illness by 12-36 hr. Within 6-72 hr of the onset of fever, an increase in total amino acid concentration above normal occurred in subjects with typical illness. Although the mechanism of these changes was not clear, Dr. Beisel's studies of adrenocorticoid secretion during tularemia^{4/} suggested that the amino acid increases following fever might have been the result of a steroid catabolic effect.

VEE vaccination at 0800 hours resulted in a loss of amino acid periodicity for 4 days following vaccination. In contrast VEE vaccination at 2000 hours resulted not only in a disturbance of periodicity lasting 2-8 days but an elevation of total amino acids 1-2 days postimmunization and depression on days 3-7. During the course of these studies of man infected

* U. S. Army Medical Unit.

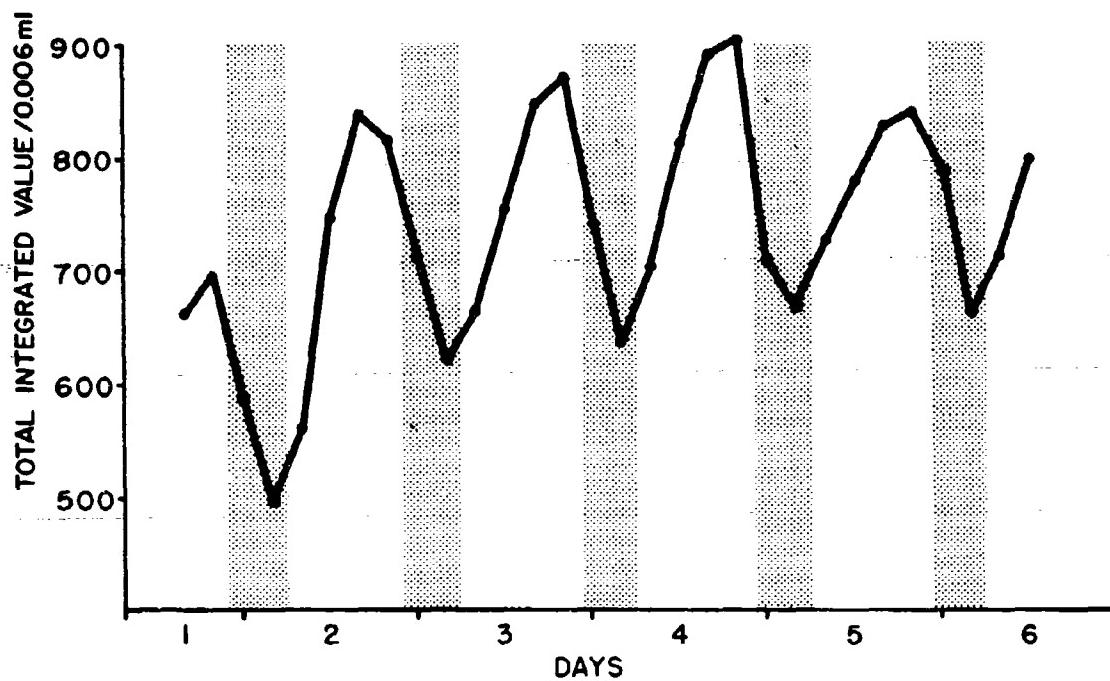


FIGURE I. CIRCADIAN PERIODICITY OF WHOLE BLOOD
AMINO ACIDS OF NORMAL MEN.

with attenuated VEE virus, marked changes in the blood concentrations of proline, glutamic acid, and glutamine were seen suggesting virus-induced inhibition of the enzyme glutamine synthetase. Whether these changes reflect the action of the virus on the brain, or are the result of viral-induced changes in other tissues remains a source of speculation. However, the finding that glutamic acid and glutamine are markedly affected, coupled with the observations that these amino acids play such an important role in neural protein metabolism and purine biosynthesis, is consistent with the available knowledge about the pathogenesis of this viral infection.

Because amino acid studies were initiated with the hope of providing a means of early diagnosis of infection, we realized that our ability to define infection-induced whole blood amino acid changes would be directly related to our understanding of what constitutes the normal in healthy young adult subjects. In addition it is hoped that these studies will answer some of the questions raised by members of the Commission when this preliminary data was presented previously.³⁷

Because of the role of amino acids in so many aspects of intermediary metabolism and the obvious myriad of controlling factors, it was necessary to investigate the possible influence of various exogenous and endogenous synchronizers.

Figure 2 shows the composite data of a 12-hr shift in the sleep-wakefulness cycle for 6 normal adult males. Changes in the rhythmicity of body temperature, the urinary excretion of Na and K, and urine volume, known parameters with endogenous rhythmicity, were measured in this study for comparison with observed changes in amino acid periodicity. Subjects were studied on a hospital ward to control environment and timing of sleep, activity, and meals. After 3 days on normal routine, a 12-hr phase-shift in their sleep-wakefulness cycle was instituted and continued for 10 days (dotted area). Following this, subjects were returned to normal habits and studied for 5 more days. The area between the dashed lines and the center section, reversed, represents the time required to reverse the sleep-wakefulness pattern. The black bars represent periods of time during which each parameter manifested a reversal of its normal periodicity. Within 24-48 hr, a rapid reversal of the normal circadian periodicity of blood amino acids was observed such that peak values were observed at 0400 rather than at 1200 to 2000 hours. Note that none of the other parameters changed as quickly or as consistently. Blood amino acids reversed in all 6 subjects. Only 4 of 6 reversed their body temperature patterns; 3 of 6, their urinary Na and urine volume; and 5 of 6, their urinary K excretion. Return to the original cycle resulted in resumption of normal amino acid periodicity within 48 hr in all subjects. The rhythmicity of blood amino acids therefore could be dissociated from that of body temperature, urine volume, and Na and K excretion, 4 parameters with known endogenous rhythmicity. This suggests that blood amino acid periodicity may be significantly influenced by exogenous synchronizers such as light and darkness and sleep and wakefulness.

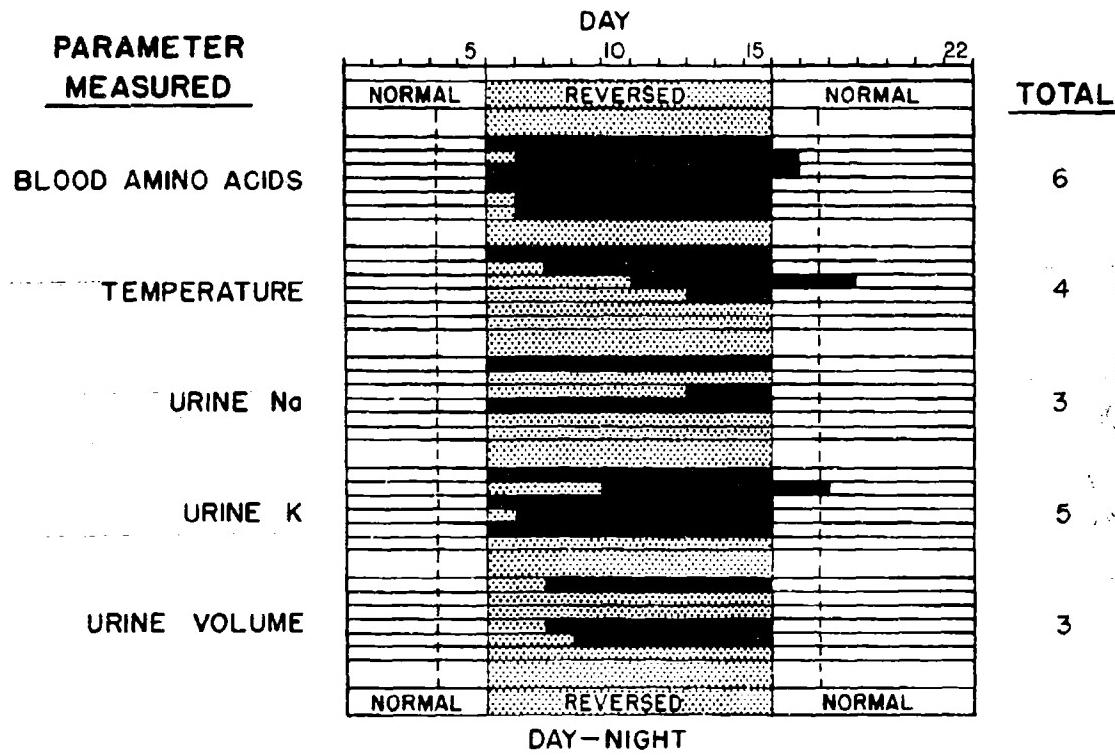


FIGURE 2. REVERSAL STUDY IN 6 MEN.

The effect of dietary protein loading (500 gm of lightly broiled beef liver containing 100 gm protein) on blood amino acids in 3 subjects is shown in Figure 3 and compared with the circadian periodicity of whole blood amino acids of the 6 normal subjects. The normal values were used to predict the periodicity of the test subjects; observed values, obtained after the test meal, were compared statistically to the predicted values. Following ingestion of the test meal in the morning, a statistically significant increase in blood amino acids was observed with peak concentrations noted 2-3 hr postingestion, demonstrating that the 500 gm of liver ingested in the morning caused whole blood amino acids to rise more rapidly than expected.

In contrast, the ingestion of the same test meal in the evening produced no significant elevation in whole blood amino acids above the fasting 2000-hour value. Rather, a decrease in blood amino acid concentrations was noted, indicating that the ingestion of a protein load in the evening did not prevent the anticipated decrease in the concentration of amino acids due to circadian periodicity.

Analysis of the single amino acid groups described previously revealed that all followed the same pattern as that obtained for their sum, with the exception of the leucine-isoleucine group. A significant increase in this group was noted regardless of the time of food ingestion. An isolated rise in the leucine-isoleucine group following protein loading reported by Frame^{5/} and others remains unexplained.

Dietary influence upon blood amino acids has been a source of considerable speculation. Our dietary studies were designed to evaluate whether dietary intake is responsible for the amino acid periodicity observed and not to determine the role of dietary protein in the maintenance of total blood amino acid concentration. This study demonstrated that although ingestion of a large portion load exaggerated the normally occurring morning increase in amino acid concentration, it did not prevent the normal evening decrease in amino acid concentration. Normal dietary protein intake, therefore, is not responsible for the circadian periodicity of amino acids which has been observed.

We also found that starvation or a period of acute exercise had no demonstrable effect on amino acid periodicity. Because of the possible relationship between peak corticosteroid secretion and the timing of minimal whole blood amino acid concentrations, this was studied in normal and adrenalectomized mice. Damping of the periodicity was observed, suggesting that the adrenal may play a role; it is obviously not the only requisite for maintenance of rhythmicity. No changes in blood amino acids were observed when sub- or supraphysiologic doses of adrenalin were given to monkeys, suggesting that catecholamine secretion does not play a major role in blood amino acid rhythmicity. We also have demonstrated that the circadian periodicity of whole blood amino acids is not the result of changes in either hemoglobin, hematocrit, plasma volume, or total white blood count, each of which demonstrates its own periodicity.

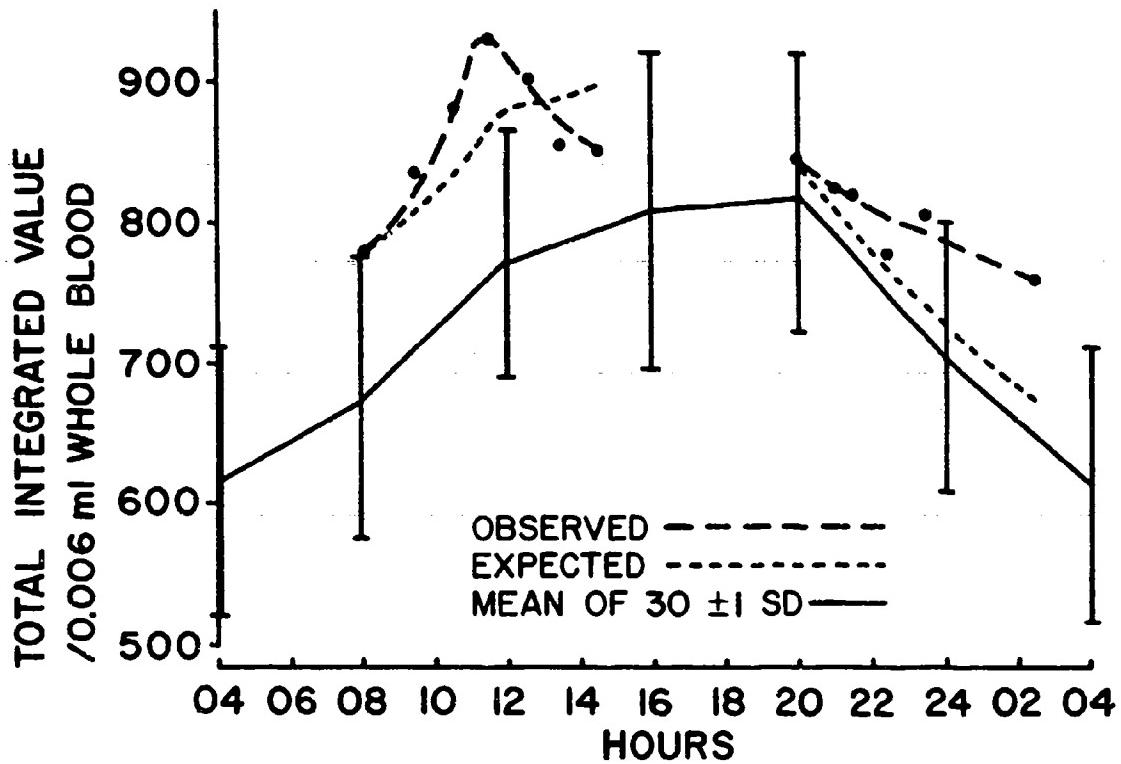


FIGURE 3. EFFECT OF A DIETARY PROTEIN LOAD ON BLOOD AMINO ACID PERIODICITY.

Despite the report in 1940 by Farr et al.^{6/} of hypoaminoacidemia in patients with pneumococcal pneumonia, few studies have been performed to evaluate amino acid changes in terms of the host's response to infection. The work of Dr. John Woodward's team^{7/} and Dr. Squibb^{8/} has stimulated renewed interest in infection-induced blood amino acid changes. Evidence is rapidly accumulating that evaluation of whole blood amino acids may prove useful in the early diagnosis of infection and may yield important clues to the biochemical and physiologic factors involved in the pathogenesis of infectious disease.

Having defined further the characteristics of whole blood amino acids in humans, we have continued to evaluate their detection and measurement as a means of early diagnosis of infection.

As part of an investigation into the efficacy of typhoid vaccine as well as to permit earlier diagnosis and control of infection, the opportunity was available to study whole blood amino acids in volunteers following oral challenge with the Zermatt strain of Salmonella typhosa (Vi strain, phage type E). This study was done in conjunction with Doctors Woodward and Hornick of the University of Maryland School of Medicine. Seventeen healthy male subjects, inmates of the Maryland House of Correction, participating on a voluntary basis were employed for this study. All subjects were challenged orally on day 0 with 10⁷ organisms. Patients who manifested symptoms were treated with either chloromycetin or ampicillin.

Figure 4 shows whole blood amino acids in 3 subjects who displayed different clinical responses to the infecting organism.

Between 1 and 7 days postchallenge a transient but significant increase in whole blood amino acid concentrations were seen in 9 of 10 subjects eventually developing typical illness. Illness was characterized by simultaneous occurrence of fever, constipation, headache, lower abdominal pain, and malaise. There were no prodromal symptoms. Significant increases in blood amino acids preceded any signs of illness by a minimum of 24 hr in each individual. Only one subject who subsequently became ill manifested no amino acid change prior to the onset of symptoms. Similar amino acid changes were not noted during the incubation period of any subject who did not manifest subsequent illness. Thus, the changes noted in the incubation period would have permitted correct prediction of the subsequent onset of illness in 16 of 17 subjects.

Fifteen subjects had decreased blood amino acid concentrations at some time following infection whether they developed clinical illness or not, but the decrease was greater in the group that became ill.

In most subjects who developed clinical symptoms, transient and intermittent reversal of normal amino acid periodicity was noted as in Case 10 (Figure 4). These changes were similar to those previously described in other infections.

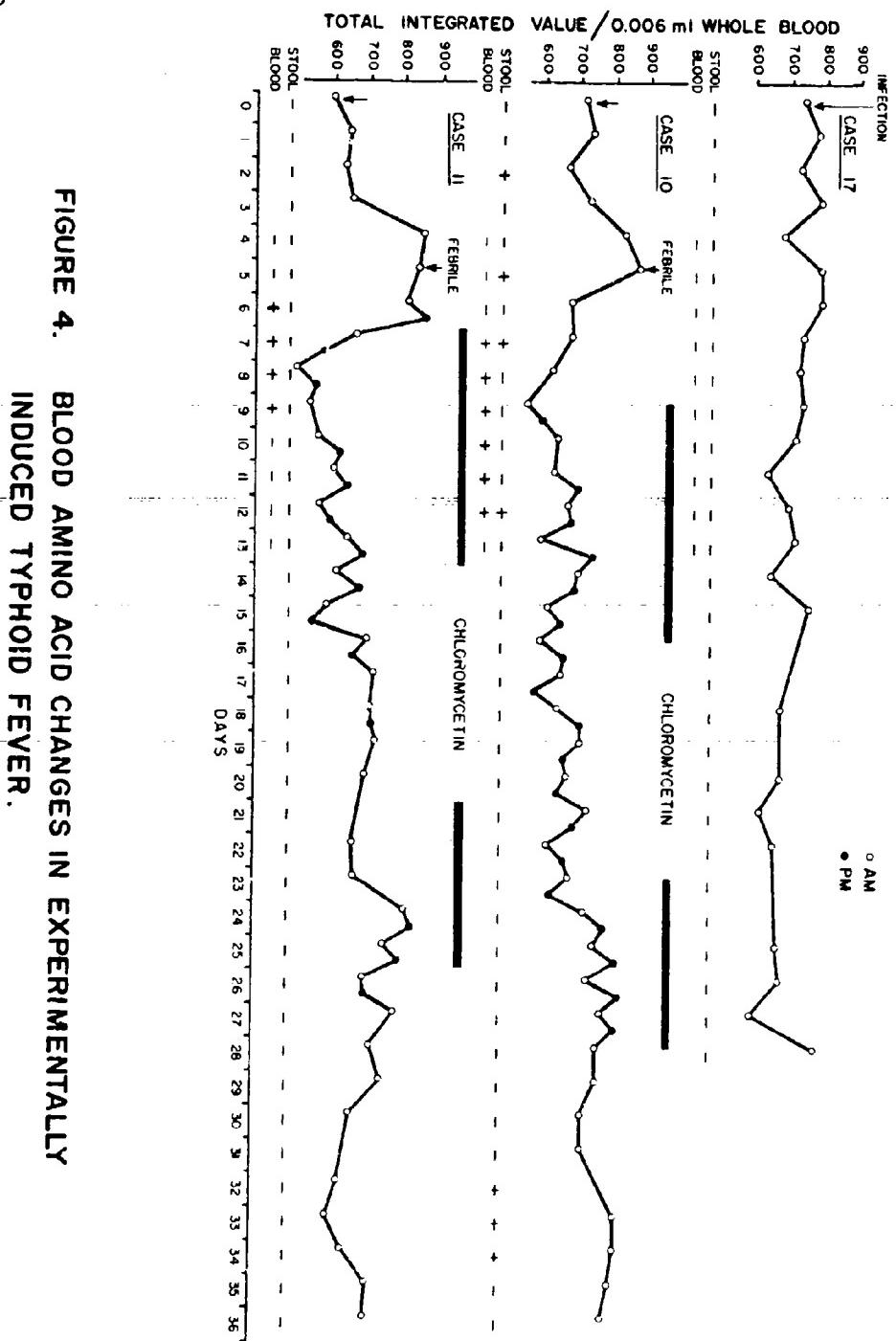


FIGURE 4. BLOOD AMINO ACID CHANGES IN EXPERIMENTALLY INDUCED TYPHOID FEVER.

Amino acid concentrations were relatively stable in Case 17 who did not develop clinical illness. Subject 10 became ill on day 5. The onset of fever was preceded by increased amino acid concentrations on day 4 as well as by a positive stool culture on day 2. Blood amino acid concentrations had already decreased prior to the initiation of chloromycetin therapy, so the effect of this antibiotic did not account for the amino acid changes observed. Subject 11 likewise developed an increased blood amino acid concentration prior to the onset of fever. In this case, neither blood nor stool cultures were positive prior to the rise in blood amino acid levels. Comparison of these subjects and the total group, therefore, demonstrated no causal relationship between the observed changes in blood amino acids and either positive stool or blood cultures.

There was no correlation with O, H or Vi antibody titers. The increased blood amino acid concentrations noted in subjects with experimentally induced typhoid fever would be of little diagnostic aid in any given patient since it is unlikely that one would seek medical attention prior to the onset of symptoms. However, in a typhoid epidemic within an institution or a community, the sampling of blood amino acids might be helpful to anticipate, in most cases, which individuals might become ill and hence permit earlier institution of therapy and an attempt at limiting spread of disease. Inasmuch as we were able to predict subsequent illness with 94% accuracy in this study, blood amino acid studies in infection may be of more than academic interest.

From our brief experience with infection-induced changes in blood amino acids it has become obvious that the practical application of this data is hampered by the periodicity of amino acids and the variability within a day, between days, and from man to man. Predicting retrospectively when infection started from a complete curve as that shown for subjects with typhoid fever is a far different task than to identify the presence or absence of infection from 1 or 2 values for whole blood amino acids in any given individual. We are in the process of solving this problem.

In Figure 5 is shown a computer-derived equation* which allows a gross estimation of normal levels of whole blood amino acids at any time of day. It is the equation for this curve which closely resembles the periodicity curve previously shown. The constant k for total integrated value, total ninhydrin-positive material, single amino acids or groups of amino acids is shown on the right. The use of this formula is presently limited by the fact that it is derived from data obtained from only 6 healthy volunteers; the validity of the equation itself as well as the spread of normal values about the curve is being tested. Should it prove valid, it would allow us to predict whether any single amino acid value regardless of the

* Mathematical analyses of all data was conducted by L. W. Gaudette, G. L. Jessup, and W. D. Foster, Biomathematics Division, Fort Detrick, using a Univac solid state II computer.

$$Y = 10^{(.139631)e^{-x/8}} + k$$

where: Y = amino acid integrated value
 x = time (hr of day / 400)
 k = constant

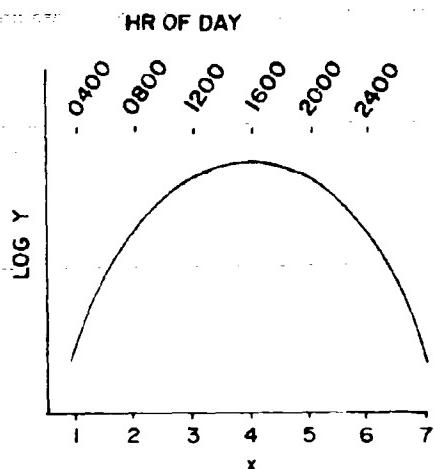


Table of "k"	
Amino Acid	Const. "k"
α -Amino N	1.33
Total	2.78
Cystine	1.74
Arg-Lys-His	1.80
Glutamine	1.585
Gly-Asp-Ser	1.91
Thre-Glu	1.645
Alanine	1.88
α -Amino Butyric	1.70
Tyrosine	1.39
Meth-Val-Tryp	1.855
Isoleuc-Leuc	1.745
Proline	1.655

FIGURE 5. EQUATION FOR ESTIMATION OF AMINO ACIDS.

periodicity or individual or group variation was normal or abnormal by comparing the observed value to that obtained from the equation. Although the use of such a formula certainly lacks the advantage of daily or twice daily amino acid levels it may endow our data with the practicality necessary for widespread use.

In general, then our data suggest that amino acid changes are seen in a variety of infections and that they constitute one of the earliest detectable infection-induced metabolic responses of the host.

Beisel et al.^{4/} have recently defined the broad metabolic changes that occur in response to infection in man. They also emphasized that the metabolic responses during clinical symptoms of a generalized infection in the human were qualitatively similar, relatively stereotypes, and not specifically related to the etiology of the infection. Similarly we found that vaccination with standard 17-D strain yellow fever vaccine, a live attenuated viral vaccine which has a lower incidence of complications than any other widely used vaccine, may result in reversal of amino acid periodicity 8 hr after immunization. The disturbances in periodicity were similar to those described for attenuated VEE virus vaccine; both are arboviruses. The sensitivity of the amino acid changes observed has been demonstrated but, of course, suggests that specificity is lacking.

The magnitude of the catabolic responses during infection have been related to the severity and duration of the illness. In addition to the wasting effects of an illness it has been shown that infection may also stimulate protein anabolism. Other studies indicate that increased glucocorticoid secretion may stimulate early hepatic protein and enzyme synthesis.^{9/}

The measurement of whole blood amino acids represents the assay of a pool of material with a continual turnover. Despite the various influences of time of day, exercise, sleep-wakefulness habits, and diet, blood amino acid concentration, in the absence of an acutely ingested protein load, remains relatively stable. In contrast, infection can produce marked changes in blood amino acid concentrations. These changes are undoubtedly related in time and magnitude to the balance between the catabolic and anabolic responses of the host to infection and most likely are the result of a multiplicity of distinct host responses reflecting the effect of infection upon a large number of enzymes and involving body protein in many organ systems.

SUMMARY

Much additional information is needed. It should be emphasized that data during infection represent actual observations. It was necessary to document the changes occurring in amino acids during infection. Now that this has been accomplished, we must show that these changes are specific for infection. Therefore future plans must obviously include

study of diseases of other than infectious origin. The ultimate hope is that refinement of the method will make it possible to identify accurately the presence of infection before the onset of disease. It is unlikely that we will be able to identify specific agents, but we may be able, at some time in the future, to identify broad categories of infectious diseases, e.g., bacterial, rickettsial and viral. Blood amino acid changes remain one of the most consistently demonstrable early biochemical indicators of infection and occur in the absence of disease or of cultural and serologic evidence of infection.

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DISCUSSION

DR. YOUNG: How did you determine the influence of protein intake on the periodicity of amino acids? I wonder if you measured blood amino acids throughout the day on a protein free diet to determine if protein feeding may shift the periodicity of blood amino acids.

DR. KLAINER: We know that a brief period of starvation resulted in no change either in periodicity or the concentration of total amino acids. Some compensatory physiologic mechanism must make this extremely constant. Large increases in blood amino acids have been described as a terminal event in almost every illness; and, in addition to our data, there is some published evidence that even a protein-free diet changes neither the periodicity nor concentration of blood amino acids. Unfortunately, the majority of published reports on total or individual blood amino acids completely disregard the effect of periodicity. Published dietary studies do not mention the time of day that protein was given. After an 8-hr fast (one might assume that test feeding was done in the morning) reports state that blood amino acids go up and then come down. Similar changes have not been sought in control subjects that have not been given the protein load. It is therefore impossible to determine from published data whether blood amino acid concentrations are influenced by the protein load given or merely by periodicity changes. We did this study specifically to determine the effect of protein on periodicity.

DR. YOUNG: Concerning your data on whole blood amino acid changes during typhoid fever, do you believe these changes involve only 1 or 2 amino acids or do all of the whole blood amino acids appear to increase and decrease simultaneously?

DR. KLAINER: In most of our experiments in which we have studied the rise and fall in blood amino acids individual and groups of amino acids appear to follow the same direction and magnitude as their sum. This obviously is not always the case, for after VEE vaccination, we were able to detect changes in proline, glutamic acid, and glutamine that did not follow the pattern of total whole blood amino acids.

DR. MacLEOD: Following trauma or surgical operations there occurs the so-called negative nitrogen balance which persists for quite a period of time and is somewhat similar to the events that occur during infection. Is there an amino acid change known to follow trauma or surgical intervention?

DR. KLAINER: There is an increase in blood amino acids following severe trauma or surgery. A difference does exist in infection, for according to our work changes in blood amino acids could be detected prior to the onset of clinical symptoms in the infectious illnesses studied by us.

DR. KNIGHT: Logic would suggest that the changes you see in amino acids are the result of infection and presumably occur because micro-organisms are growing somewhere within the body. While this change in amino acids may provide valuable lead time for diagnosis, I am curious as to the relationship of this change to the infectious process.

DR. KLAINER: The exact mechanism for the amino acid changes is not known. We find that live plague vaccine caused amino acid changes that were considerably more prominent than those we detected after vaccination with killed organisms. Dr. John Woodward's early work on tularemia in mice led to the suggestion that depression in whole blood cystine was due to the selective utilization of that amino acid by organisms growing within the spleen, since cystine is an important and essential amino acid for the growth of Pasteurella tularensis. Our own studies of tularemia in man reflected more generalized changes in whole blood amino acids, changes that were not limited to a single amino acid. This would imply that we are measuring a change in host metabolism rather than one specifically due to the metabolism of the microorganism itself.

DR. KNIGHT: I wonder if the outpouring of amino acids into blood represents a breakdown of protein. Can amino acid changes be related to specific anabolic or catabolic changes?

DR. KLAINER: I wish I could answer your question. However it has been possible to correlate changes noted in blood amino acids during tularemia with the studies of nitrogen balance by Colonel Beisel. In some cases decreased whole blood amino acids occurred during periods of positive nitrogen balance, while increases in whole blood amino acids occurred during periods of nitrogen catabolism.

DR. KNIGHT: Did none of the typhoid patients challenged with viable organisms show changes in amino acids if they failed to become ill?

DR. KLAINER: None of them.

DR. KNIGHT: Is it true, Dr. Hornick, that a number of the asymptomatic patients had positive stool cultures?

DR. HORNICK: True. No asymptomatic patient however had a positive blood culture.

DR. KLAINER: In patients who became sick it was frequently possible to correlate the decrease in amino acids seen following the onset of illness with positive blood cultures. A lesser degree of fall in whole blood amino acids was seen in patients who were only mildly ill many of whom did not have positive blood cultures.

DR. T. E. WOODWARD: It would be interesting to determine if whole blood amino acid changes might occur in the occasional patient who has a positive blood culture without evidence of clinical illness.

DR. JOHN WOODWARD: The question was raised earlier as to whether some amino acids were depleted or changed selectively. In some of our earlier work we did find a depletion particular in cystine. It seemed to disappear, at least by any methods available to us. My colleague, Dr. Sbarra, and I used to argue as to the reasons for this. Although the P. tularensis organism was selectively using cystine and does require cystine in its nutritional requirements, I've never felt such an explanation provided the entire answer to the mechanism of cystine depletion. I don't really think that the organism was selectively making use of cystine to the extent that would cause a total depletion from the blood stream. One can roughly divide the possibilities which result in increased plasma amino acid concentration into 4 categories. First, there could conceivably be increased endogenous synthesis of amino acids which seem unlikely during infection. Second, there could be a decreased movement of amino acids across the cell membrane. Third, there could be a decrease in incorporation of free amino acid into protein, and fourth, an increased rate of protein degradation could raise whole blood amino acids. I wonder if it would be possible to help narrow down these possibilities by using N^{15} -labeled amino acids and measuring their rates of incorporation into protein.

DR. KLAINER: All of your suggestions are good ones. Studies of this nature must be planned for the future. At the present time our work has involved an attempt to determine if infections induce changes in blood amino acids in patterns that we could differentiate from changes in the normal host. A search for mechanisms of change obviously remains to be done.

DR. GRAY: We studied the uptake of S^{35} into plasma proteins in rabbits that had been infected with tularemia; noninfected rabbits were used as controls. When studied 24 hr after rabbits had been infected, there was approximately a 3- or 4-fold increase in the uptake of methionine into plasma protein. Later, in a group of 3 chimpanzees we studied the uptake of labeled methionine during infection with VEE. Its uptake 24 hr after infection was also increased above the value that we had measured during artificially induced fever alone, so apparently in the presence of either tularemia or VEE there is an increased uptake and possibly an increased turnover of methionine.

DR. INGBAR: What is your thinking with regard to the adrenal rhythmicity in relation to the diurnal variation of amino acids? As I understood it, some diurnal rhythmicity persists after infection and it is my understanding that the adrenal rhythm is lost for a time. Is that correct?

DR. KLAINER: The rhythm of amino acids in blood wasn't actually lost, but it was markedly dampened in adrenalectomized mice. I call it dampened because adrenalectomy narrowed the difference between morning and evening values. In addition, the fact that the low values in blood amino acids and the peak values in normal adrenocorticoid secretion both occur early

in the morning certainly suggest that there's an interrelationship. I think this is worth looking into more closely, especially in infection.

DR. BESSMAN: Could you explain the term "Total Integrated Value" on the Y-axis of your slides? How does this determination compare to a value in gamma/100 ml?

DR. KLAINER: The term Total Integrated Value is a semiquantitative term which represent the total integrated area under the amino acid curve on a densitometer; it is possible to change this back into $\gamma/6 \mu\text{L}$ or γ/ml of blood. We believe that the Total Integrated Value can be used directly for estimating changes in whole blood amino acids in serial studies; thus for our purposes, one does not have to convert it to another figure. Such changes can be substantiated by measuring total plasma ninhydrin-reacting material and we also have been able to check our values by occasional runs on an amino acid analyzer. Because of time and expense, it is obviously impossible to obtain a formal analysis on every sample we collect in a study. Rather, we have employed our paper chromatographic method to be able to study up to 100 samples a day, week after week. Obviously this sacrifices quality to get quantity. This is a rapid method which allows us to pick up changes and differences in single subjects from hour to hour.

DR. BESSMAN: On the basis of these figures do you calculate that the total diurnal variation in terms of baseline value or mean daily value would be somewhere around $\pm 15\%$?

DR. KLAINER: In our normal subjects there was an average of about 40% difference between morning and afternoon values.

DR. BESSMAN: This is a paper chromatographic method, so that I assume the variation on duplicates is quite large, as ours is. I would raise the question that the major changes in the blood amino acid pattern during infection is approximately equal only to the total range of the diurnal variation. When this is the case, it depends upon the time of day you sample to determine whether or not you can predict an impending infection in a particular individual. On the other hand, the graphs that you presented here in the patients with typhoid fever or subjects without any trouble from infection often showed no p.m. values; in other cases there was a considerable fluctuation which apparently was due only to p.m. and a.m. differences.

DR. KLAINER: Right. I can answer your question in two ways. The error of our method is roughly between 5 and 6% and this has been well documented on hundreds of samples. This is relatively small methodologic error and has been checked by a number of other procedures. We have considered changes to be significant in infected subjects only if they exceeded the possible change for diurnal variation plus the error of the method. I think we've been on the safe side in describing significant and nonsignificant changes during infection.

DR. BESSMAN: Do you mean that 5% between any pair of duplicates is the maximum difference you're going to get? This to me is a very critical question as to whether or not you can describe a change in a particular patient.

DR. KLAINER: If we take duplicate samples and study a hundred pairs the average error was 5.7%. I must emphasize that the absolute concentration varies from person to person and even varies considerably in the same man from day to day at any time of the day we measure it. This fact is obvious from the graphs we showed. The literature also gives an exceedingly wide range for normal values in humans. In addition to having control subjects who are not exposed to infection, we attempt to use each man as his own control and whenever possible get at least 3-5 days of sampling prior to infection to permit us to estimate the variability of each man with respect to variability from day to day and variability due to diurnal or circadian change within single days. This amount of variability is taken into account when we analyze our data from the infected days in a statistical comparison to data obtained in the same individuals prior to infection. We do anticipate real difficulty as you point out if we would attempt to take single values from a subject and determine from them alone whether the individual is within the normal or abnormal part of a serial change.

DR. KNIGHT: I might have missed it but what was considered the incubation period in the typhoid studies?

DR. KLAINER: The incubation period in typhoid fever was about 5 days. There was a slight peak of amino acids rising on days 3 or 4 which stayed up until the first day of fever and then dropped to abnormally low values. Again this fall was greatest in people who became ill, but some fall was also seen in people who did not become ill.

DR. JOHN WOODWARD: Do you have any information concerning amino acid changes in illness other than those due to infection?

DR. KLAINER: No. Before we can call any of this work specific for infection it will be necessary to screen a number of diseases of non-infectious origin.

AMINO ACID CHANGES IN EXPERIMENTALLY INFECTED CHICKS

Robert L. Squibb, Ph.D.*

Our principal objectives have been to observe biochemical changes in selected avian tissues during Newcastle disease virus (NDV) or avian tuberculosis (TB) infections, and to record whether changes that occur in tissue components have magnitude, are reproducible and are specific for a particular infection. Findings which meet these requirements are further evaluated for possible use as diagnostic tools.

The experimental models employed will be outlined first. We are using 4-6 week old cockerel chicks of known breeding. It is felt that the chick has a number of distinct advantages over other laboratory animals in that it can be obtained reasonably disease-free at time of hatch. Further, present-day knowledge of the nutritional requirements and pathology of the chick is without doubt superior to that of other laboratory animals. There is also the added advantage that the chick is a natural host to several viral and bacterial infections which are closely related to measles, mumps, parainfluenza and canine distemper;^{1/} while Mycobacterium tuberculosis, avian strain, produces a chronic, fatal disease which is characterized by tubercles whose development essentially parallels that observed in man.^{2/}

By using a growth curve developed from a standard reference diet it is possible to detect when a particular control group does not meet its genetic potential for growth. Failure to do so is attributed to the presence of an unforeseen stress either of exogenous or endogenous origin and which, therefore, could confound biochemical findings. From the time of hatch all chicks are reared on known references or experimental diets. All experiments are carried out in isolated air-conditioned rooms wherein care and management are carefully controlled.

We have used the same strain of NDV for the last 9 years; this is the "Grun" strain named after Dr. John Grun (Rutgers University) who maintains and prepares our inocula. By varying the dilution of the NDV inoculum, mortality can be adjusted from 0-65%, with better than 99% confirmed infections. For our TB studies a Rutgers culture, M. tuberculosis, avian strain Kirchberg, is used; it is prepared and standardized by Dr. Morris Solotorovsky of the Bacteriology Department.

With the Grun strain of NDV the infection cycle follows a well-defined sequence (Table I). The first 72 hr postinoculation are termed the incubation period, during this stage there are no apparent effects on body weight or feed and water intake. The next 72 hr are the period of active

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TABLE I. NEWCASTLE DISEASE VIRUS CYCLE

PERIOD	DAYS	
	Duration	Postinoculation
Incubation	3	3
Active involvement	3	6
Initiation of recovery	8	14
Recovery	20	34

involvement during which the greatest proliferation of the virus and rise in antibody titer occurs and nearly all the mortality is recorded. Initiation of recovery begins approximately 144 hr postinoculation. Depending upon the level of inoculum used, clinical symptoms of the birds at this time range from none to paralysis in approximately 35% of the survivors.

The biochemical parameters used are those involved in protein metabolism,³ a process recognized as being affected by disease (Figure 1).

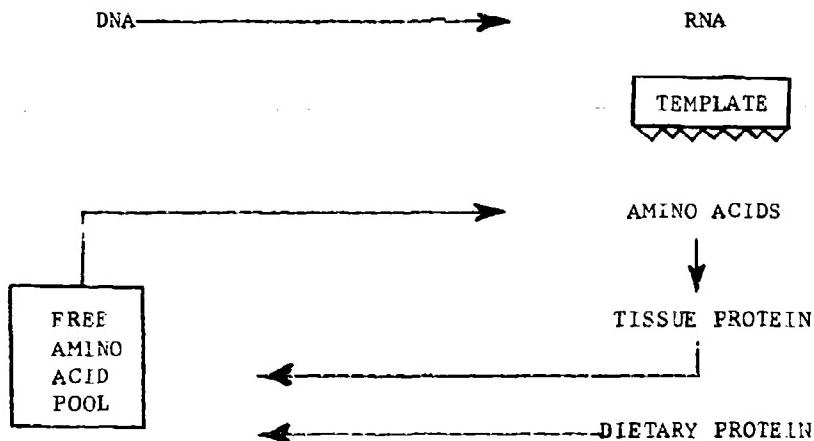


FIGURE 1. SCHEMATIC REPRESENTATION OF PROTEIN SYNTHESIS.

The parameters, DNA, RNA, total protein, and some 10 free amino acids, represent the genetic components of the cell as well as the materia prima for protein synthesis. All biochemical determinations are made on individual samples for which some 8-10 individual chicks are

sacrificed for each point on a curve. Additional groups of birds are inoculated and observed over the entire cycle of the NDV infection in order to establish the per cent mortality or intensity-of-involvement index. With TB infection a disease-involvement index is obtained from a formula wherein liver weight x number of tubercles per unit area is divided by the body weight of the bird.^{4/} It is not unusual for most experiments to require anywhere from 200-500 individuals. Since a 4-6 week old chick is in the stage of very rapid growth, changes in the previously described biochemical parameters are assumed to reflect treatment interactions within the anaabolic phase of protein metabolism.

Earlier work in our laboratories directed toward the standardization of NDV and TB models showed that a number of stress factors had significant effects on tissue levels of free amino acids. For example, as the severity of NDV infection increased (Table II) there was a significant linear depression of the free amino acids in the liver, whether the values were calculated

TABLE II. EFFECT OF DEGREE OF NEWCASTLE DISEASE INVOLVEMENT ON THE FREE AMINO ACID POOL

SYMPTOM	WEIGHT AMINO ACIDS mg/gm wet liver							
	Lys.	His.	Arg.	Asp.	Acid	Ala.	Val.	Leu. ^{a/}
Noninfected	.91	.53	.80	1.50		.91	.68	.95
NDV-Infected								
None	.58	.44	.54	1.11		.66	.47	.71
Respiratory	.51	.48	.49	1.23		.67	.41	.59
Partial paralysis	.53	.39	.53	1.06		.67	.42	.59
Full paralysis	.46	.47	.46	1.15		.62	.35	.50
Dying	.49	.35	.43	.77		.56	.39	.52
Dead	.28	.37	.30	.55		.62	.30	.31

a. Leucines combined.

in terms of mg/gm of fresh tissue or mg/mg DNA.^{5/} It was apparent that histidine, alanine, and, to a lesser extent, valine, were least affected by the intensity of the infection. Avian TB likewise depleted the liver's free amino acids.^{4/} However, there is a contrast; in the NDV chicks the free amino acid pool is seldom if ever depressed to critically low levels even when the bird is dying, whereas in the case of TB pools depletion may reach levels below the sensitivity of the chemical procedures to quantitate many of the amino acids.

It is obvious from these data that one must use statistical procedures which employ grouping by outcome in order to study interactions of nutrition and/or environment with infection. This is particularly true during the recovery stage of infection.^{6/}

In many cases during certain phases of a disease cycle there is a reduction and even a cessation of food intake which can be directly related to the intensity of disease involvement. Because this dietary interaction is confounding it became important to investigate to what extent a reduced intake of food might affect free amino acid levels. A previous paper⁷ showed that diet restriction was the major single influence on a depression of nitrogen retention during the active involvement stage of NDV. In this respect, the findings in the chicken were similar to those observed by Beisel⁸ in man. As shown in Table III, starvation and NDV infection per se caused different reactions among the individual free amino acids.

TABLE III. INTERACTION OF STARVATION AND NEWCASTLE DISEASE VIRUS OF THE FREE AMINO ACID POOL

TREATMENT	WEIGHT OF AMINO ACID						
	Lys.	His.	Arg.	Asp.	Acid	Ala.	Val.
mg/gm wet liver							
Control, <u>ad lib.</u>	1.08	.46	.91	1.23	1.36	.82	1.22
Control, starved	1.18	.41	.89	1.17	1.37	.80	1.16
NDV, starved	.87	.39	.71	1.06	1.11	.68	.99
mg/mg DNA							
Control, <u>ad lib.</u>	.60	.26	.51	.69	.76	.46	.69
Control, starved	.53	.18	.40	.52	.61	.36	.52
NDV, starved	.35	.16	.29	.44	.4	.30	.41

a. Leucines combined.

On the basis of per gram of fresh tissue, starvation increased the level of lysine with little effect on the rest of the amino acids. On the other hand, infection significantly reduced all the free amino acids. When calculated in terms of DNA, as a known unit of reference, it became evident that lysine was principally affected by the disease, while in the other amino acids both starvation and disease caused a significant depression of values. These apparent differences are attributed to the fact that starvation increases the quantity of DNA/m of tissue.^{5/} In other trials the feeding of diets containing deficient-to-surfait quantities of lysine or a complete protein to birds severely infected with either NDV or TB resulted in higher mortality and lower levels of free amino acids in the livers of the imbalanced groups.^{4,9}

Having established these baselines, attention was turned to biochemical changes during early infection or the incubation period of disease. As mentioned previously, there were no apparent effects on clinical appearance, body weight or food intake during this part of the disease cycle. In order to observe the earliest possible biochemical changes during a disease cycle a technique of around-the-clock sampling was used. Starting at the time of inoculation, or 0800 hours, 8-10 chicks from each treatment group were sacrificed at 4-6 hr intervals for the entire 72-hr incubation period of NDV disease. Samples of serum, pectoral muscle and liver tissue from both control and infected chicks were analyzed for nucleic and free amino acids. As shown in Figures 2 and 3, significant diurnal changes in levels of free amino acids occurred in both control and NDV-infected tissues. The magnitude of the oscillations within a single 24-hr period ranged from 20-60%/gm of fresh tissue or per unit of DNA. In the controls, the patterns of oscillations, i.e., the peaks and troughs, of each of the 8 amino acids were quite similar, especially in the liver. In the NDV-infected chicks the changes in free amino acids were of the same magnitude but there was a greater variability in the patterns of the oscillations, particularly in muscle and serum. Of greater significance, perhaps, was the phenomenon of "desynchronization" wherein the peaks and troughs of the oscillations in the NDV tissues in relation to light-dark periods changed from that of the controls and the linear component of the slope of the liver free amino acids declined significantly. Subsequent unpublished data from our laboratories have shown that the patterns of diurnal oscillations of amino acids were highly reproducible under controlled laboratory conditions. These findings in the chick are similar to those observed by the Fort Detrick workers.

The fact that control patterns of free amino acids were reproducible (Klainer, p. 13 this report) and that diurnal oscillations were of considerable magnitude suggested the need to know whether individual free amino acids bore a relationship to one another; in other words, how constant is the "makeup" of a free amino acid pool and is it affected by the observed diurnal fluctuations of the pool itself? Further, would the within-pool ratios of amino acids have value for the early diagnosis of disease?

It is recognized that in the course of determining the free amino acids of a tissue the results are representative of many different proteins. Fortunately, in the case of the liver, parenchymal cells comprise approximately 85% of the tissue. Therefore, free amino acid extracts of this tissue are fairly representative of a single type of cell. It can be assumed that in a normal rapidly growing individual, diet intake and tissue metabolism are organized to maintain the *materia prima* in proper ratio for the code requirements of parenchymal tissue. Data supporting this hypothesis are presented in Figure 4. It can be seen that in spite of the wide daily fluctuations in the free amino acid pool of both control and infected groups there is a remarkable constancy between the ratios of individual free amino acids. The patterns of the pool oscillations, however, are desynchronized by the infection. In other words, the peaks and troughs in the diseased animals do not correlate with the controls or clock hours.

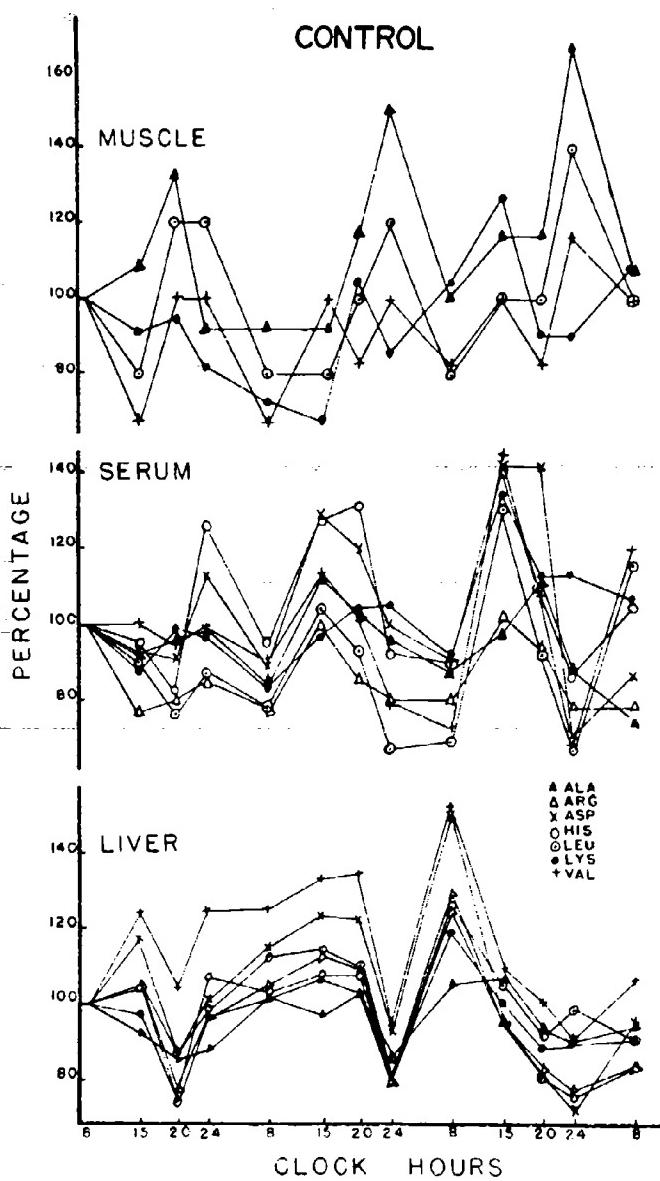


FIGURE 2. DIURNAL OSCILLATION OF FREE AMINO ACIDS IN MUSCLE, SERUM AND LIVER OF CONTROL CHICKS.

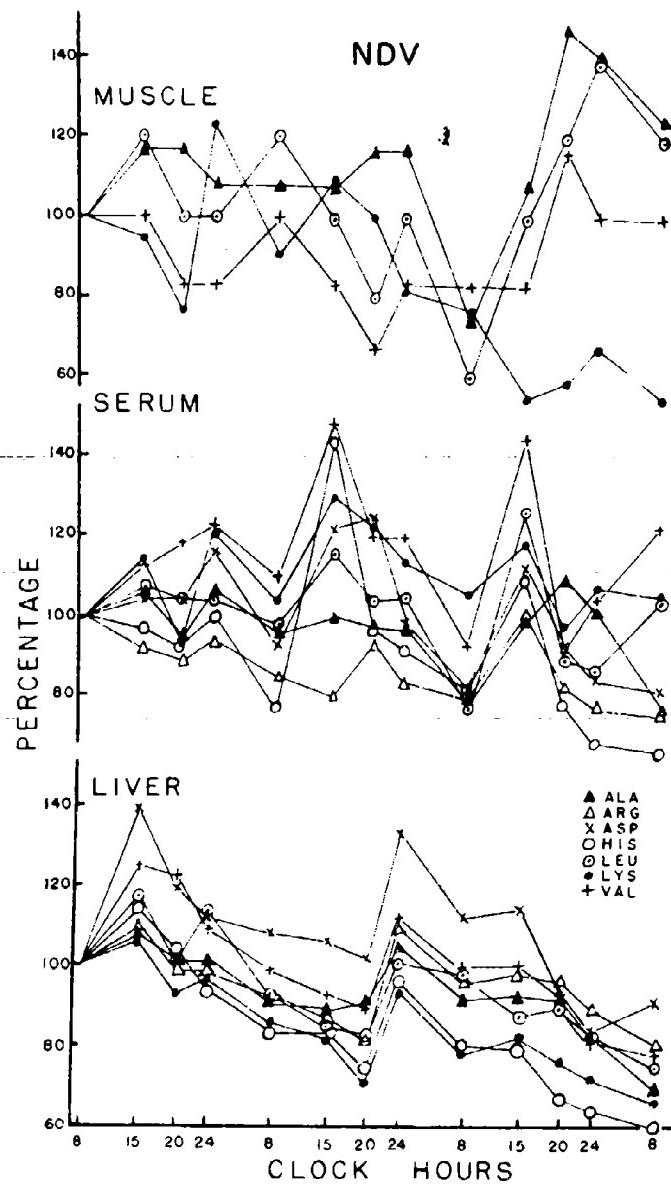


FIGURE 3. DIURNAL OSCILLATION OF FREE AMINO ACIDS IN MUSCLE, SERUM AND LIVER OF CHICKS INFECTED WITH NEWCASTLE DISEASE VIRUS.

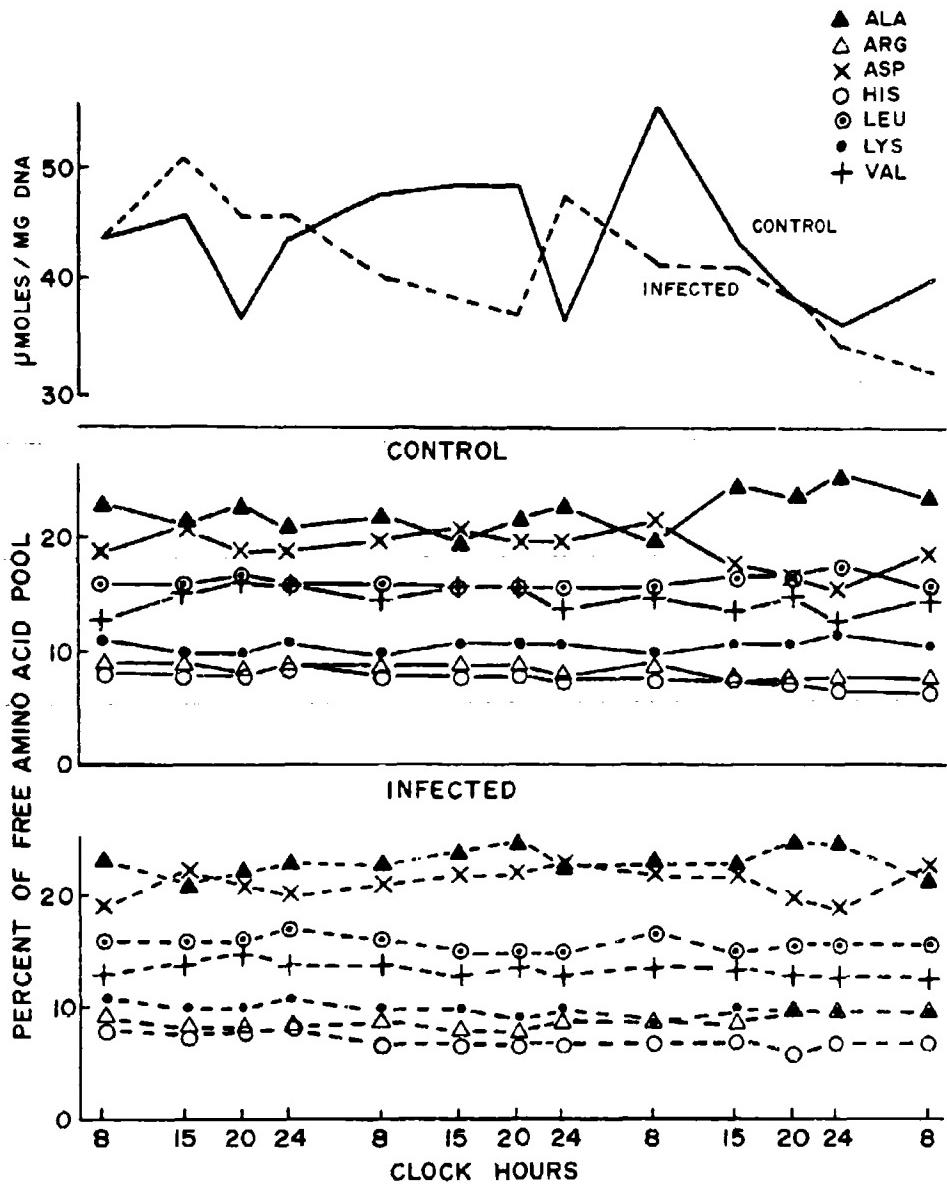


FIGURE 4. EFFECT OF NEWCASTLE DISEASE VIRUS ON THE FREE AMINO ACID RHYTHM PATTERNS AND WITHIN-POOL RATIOS IN AVIAN LIVER TISSUE.

In further study of this "constancy" phenomenon, amino acid contents of the livers of control groups of some 11 experiments observed over a 3-year period were reexamined for their within-pool ratios. It is apparent from the data of Table IV that when compared to control values, extremes of dietary imbalance and environmental temperatures (which were recorded for 24-96 hr periods) failed to change the within pool ratios in the liver.

TABLE IV. EFFECT OF STRESS ON FREE AMINO ACID RATIOS IN AVIAN LIVER

FREE AMINO ACID	CONTROL (\pm SE)	PER CENT OF AMINO ACIDS			
		4 x dietary lysine requirement	High environmental temperature	Avian TB	NDV
Lys.	11 \pm .11	12	11	4	8
His.	6 \pm .41	7	6	8	8
Arg.	8 \pm .18	8	7	5	7
Asp. acid	20 \pm .83	18	20	36	30
Ala.	25 \pm .89	25	26	26	24
Val.	13 \pm .42	14	13	12	10
Leu.	17 \pm .43	16	17	9	13
TOTAL	100	100	100	100	100

a. Values from 11 experiments comprising approximately 200 individuals.
 b. Leucines combined.

On the other hand, the presence of TB or NDV significantly changed these ratios. While there were differences between the viral and bacterial infections, further experimentation is necessary to evaluate their specificity. However, in both infections, lysine was the amino acid ratio most affected. In TB the ratio of aspartic acid to the other amino acids increased while that of the leucines decreased. Little or no effect was apparent on the alanine and valine ratios. With NDV, on the other hand, histidine and aspartic acid increased while the leucines and valine decreased. The ratio of valine, however, remained constant. Such constancy within such critical ratio shifts is worthy of further evaluation. The foregoing data indicate a need to test the hypothesis that any stress that effects a severe depression of growth may affect significantly within pool ratios or any stress that affects within-pool ratios in certain strategic tissues will affect growth. Failure of an amino acid pool to meet code requirements for synthesis could be the biochemical characterization of many dietary imbalances.

It now becomes obvious that the magnitude and continuing reoccurrence of diurnal oscillations of free amino acids and the sensitivity of their patterns to endogenous and exogenous stress poses problems for the interpretation of research results. Figure 5, schematically presented from

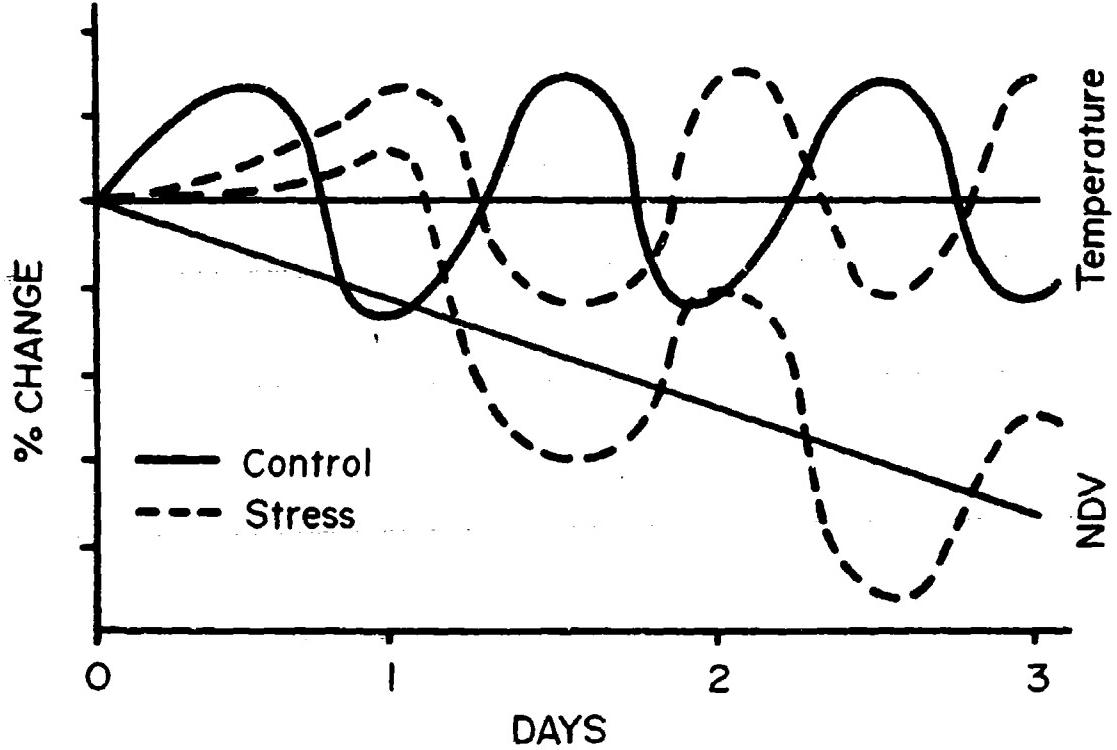


FIGURE 5. EFFECT OF HIGH TEMPERATURE AND NEWCASTLE DISEASE VIRUS STRESS ON THE LINEARITY OF DIURNAL PATTERNS OF FREE AMINO ACIDS.

actual experiments, may serve to illustrate some of these problems. Here it is evident that stress effects have (1) resulted in a desynchronization in relation to control values and clock hours without changing the slope of the linear component, and under these conditions it is apparent that failure to standardize sampling hours could result in significant treatment effects being completely reversed; and (2) in the case of a severe NDV infection one sees an immediate initial desynchronization of values which over a sufficient time interval reaches unmistakable significance because of a continuing decrease in the linear component of the fluctuations. While not fully understood at present, the phenomenon of "desynchronization" may prove to be a sensitive tool for stress evaluation. This is not to imply that this phenomenon is detrimental, it may in fact be a normal initial reaction.

Since our previous studies used constant lighting, it became important to compare continuous lighting with a 12-hr light-dark regimen. The data shown in Figure 6 leave little doubt that a 12-hr light schedule is a powerful "synchronizer" which has definite effects on changes in various tissue components during an NDV infection. Under both lighting systems body weights and tissue protein content follow expected patterns for an NDV infection, but here the similarity ends. Liver weights of the controls, which under constant light show a linear increase throughout the 72 hr of the experiment, now have distinct diurnal oscillations under the 12-hr light schedule. Under constant light the significant increase in the liver weights of infected birds, which is known to correlate with an increased nitrogen retention^{1/}, was held in synchronization with the controls by the light-dark regimen for the first 32 hr postinoculation. Thereafter NDV values were higher than the controls but remained in synchronization to clock hours. Liver DNA, which under constant light was immediately desynchronized in the NDV tissues, was held in synchronization by the 12-hr light regimen except for a brief period postinoculation. On the other hand, RNA which is depressed by the infection in animals conditioned to constant light, was held in synchronization by the 12-hr light regimen throughout the incubation period of the NDV, in spite of the contribution of viral RNA (Figure 7). The free amino acid pool in the liver followed patterns similar to the RNA. Further analysis of the patterns of the oscillations showed that the change in lighting regimen from 24 to 12 hr also caused a significant shift in the peaks and troughs of DNA, RNA and the free amino acid pool in both control and infected birds. In general, the foregoing data would indicate that disease effects become more reactive under constant lighting. This may be due to the compounding of the stress environment, e.g., the stress of constant light plus the stress of disease.

SUMMARY

For the interpretation of biochemical data it is important that the stage of the disease cycle and the degree or intensity of involvement be defined. Further, an estimate must be made of dietary interactions,

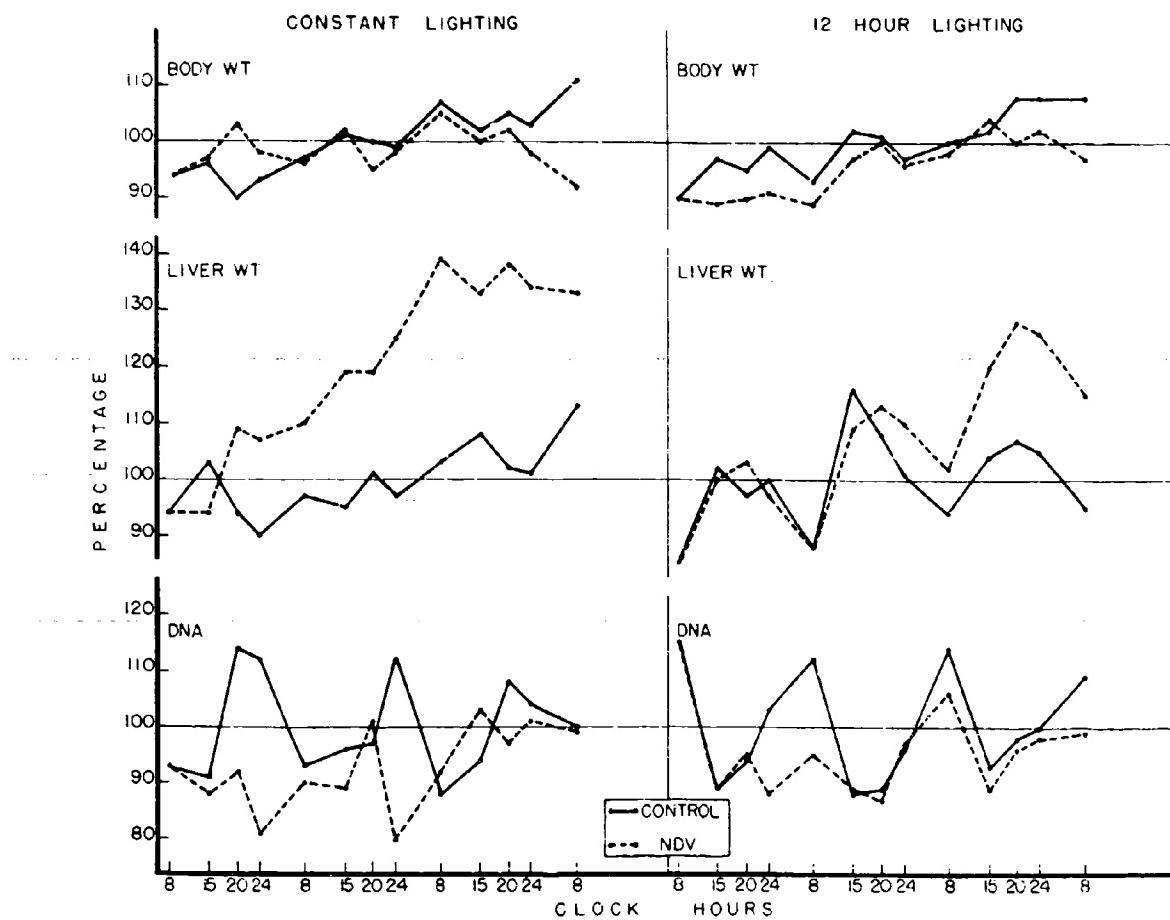


FIGURE 6. EFFECT OF LIGHTING REGIMENT AND NEWCASTLE DISEASE VIRUS ON DIURNAL PATTERNS OF BODY AND LIVER WEIGHTS AND LIVER DNA.

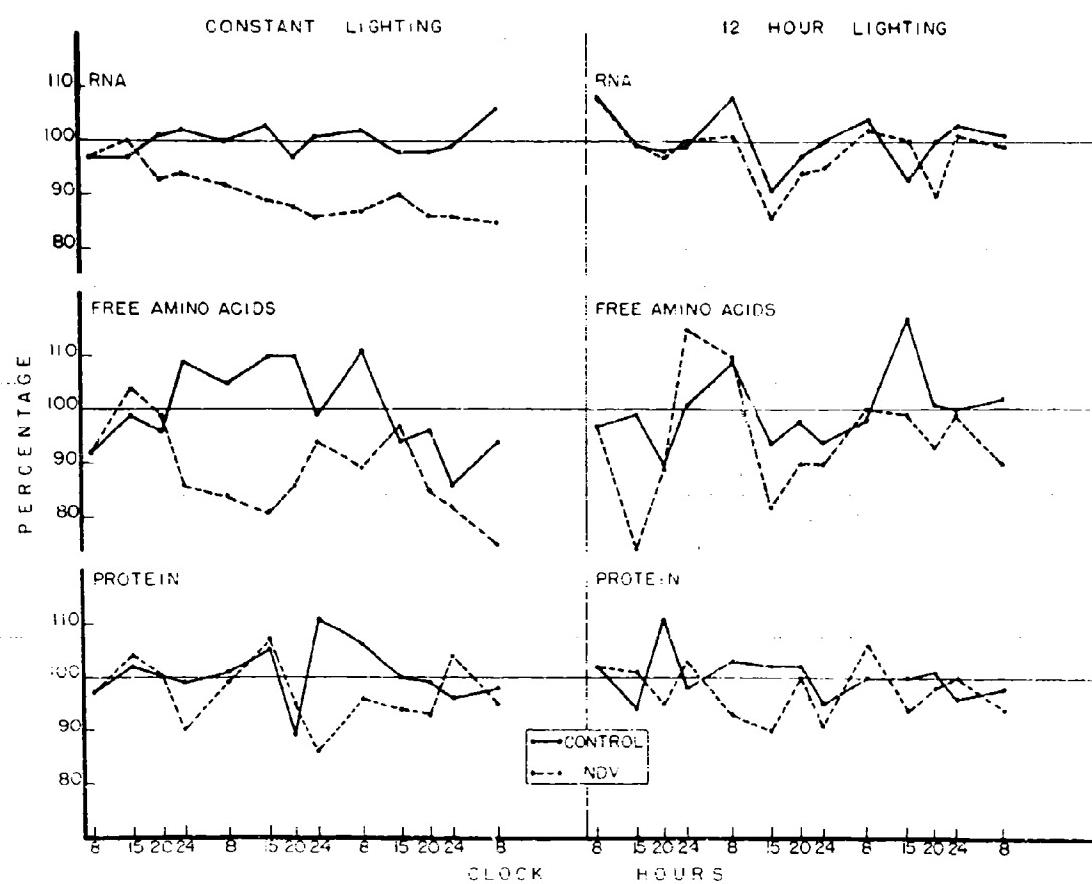


FIGURE 7. EFFECT OF LIGHTING REGIMEN AND NEWCASTLE DISEASE VIRUS ON DIURNAL PATTERNS OF RNA, FREE AMINO ACIDS AND TISSUE PROTEIN IN AVIAN LIVER.

especially where varying levels of starvation may be a significant part of the disease cycle. Studies related to the biochemistry of infection must use controls that are true controls; trials cannot be just comparisons of animals with and without a specific treatment.

The greatest challenge lies in understanding the dynamics of intact systems. Our avian and biochemical models leave little doubt that daily oscillations or fluctuations of tissue constituents occur with unbelievable magnitudes and that these are normal reflections of metabolism in the intact system. Under carefully controlled laboratory conditions most oscillations we have observed follow highly reproducible patterns which can be "mapped." The hypothesis that interrelationships of such patterns under normal and stress conditions can form the basis for exacting diagnostic procedures deserves thorough testing.

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DISCUSSION

DR. ZAMECNIK. You described a 30% increase in liver weight over a 24-hr period in the chick during NDV infection. During the same period of time the quantity of protein in the liver remained relatively constant. Do you imply that the increase in liver weight was due to an accumulation of lipid, water, or some other substance?

DR. SQUIBB: We have found moisture to be 70% and total lipids 38.4 mg/gm in control livers and 9% and 38.0 mg/gm in NDV livers. These tissue components, therefore did not contribute to the increase in liver weight. We do know, however, that the increase correlates with an increase in nitrogen retention. Other biochemical changes remain to be studied.

DR. ZAMECNIK: NDV is an RNA virus and it is conceivable that the virus might be making protein at a rate faster than the production of animal protein. Do you think that appearance of new, virus-detected protein could be related to the low level of endogenous amino acids?

DR. SQUIBB: This is a possibility. I should point out that during the initial increase in liver weight, virus titer increases only about 7% which would indicate that the production of virus protein is too limited to account for the increase in liver weight. The mechanisms behind these changes must be resolved. What is intriguing is the fact that a 12-hr lighting regimen held the rhythm patterns of the NDV liver weights to those of the controls until the latter stages of the incubation period of the virus.

DR. BESSMAN: I am also interested in the question Dr. Zamecnik raised. The observations concerning change in the size of the liver are extremely important. If such changes in liver size are not due to water which is easy to transport, they must be due to some large molecule. Changes certainly cannot be ascribed to changes of great masses of small molecules, because then an enormous osmotic effect would be present which should cause movements in water. So one must look at the energetics of this. If there is in fact no change in water one must ascribe the 30% change in liver mass to an increase in solids.

DR. SQUIBB: Well, the energy here is enormous. We have observed within a day changes in DNA to be as much as 25% and have attempted to evaluate the liver weight problem by establishing ratios of protein in terms of DNA and RNA. Such data show that protein synthesis increases significantly during the early stages of the infection.

DR. BESSMAN: If you are using the same frame of reference for two observations in 2 different situations, the ratio of the 2 should always be the same. As I recall you had changes in the amino acid ratios between starved and control animals. These ratios were also different when you compared them to the wet weight or to the mg of DNA. If both the changes in weight and the changes in DNA are saying the same thing the ratios should have been the same. I wonder, how valid are the changes between groups if they are all done in different animals and show such remarkable discrepancies? DNA is assumed by most people to represent the number of cells in the organ.

DR. SQUIBB: This assumption is not proving to be valid.

DR. BESSMAN. It is not a good assumption but is probably the best reference we have. Nevertheless it still remains an essential point that when a large molecule disappears, major changes will occur. Changes like that should involve the movement of water. Otherwise you must postulate that the rate of synthesis increases enormously for a short time and then the rate of degradation increases enormously.

DR. NEWBERNE. Does the virus proliferate in the liver?

DR. SQUIBB. Yes, this is one of the principal tissues involved in the proliferation of NDV.

DR. NEWBERNE. What is the spontaneous feeding pattern in the chick? I wonder if this influences the cyclic activity you observe.

DR. SQUIBB. We see diurnal oscillations similar to those described by Dr. Klainer, even during starvation which indicates that dietary intake doesn't effect the diurnal oscillations of plasma amino acids. In regard to eating patterns under constant lighting, chickens will roost at about 1900 hours and unless disturbed will remain so until 0500 hours the next morning. Any disturbance will awaken the animal; they usually go to the feed trough and eat before returning to roost. Another problem is that the chicken is not like a rat in relation to the absorption of nutrients following dietary intake. The chicken fills its crop; I woundn't even hazard a guess on the relationship between crop emptying in the chicken and the absorption of amino acids into the blood stream.

INFLUENCE OF BACTERIAL INFECTION ON SERUM ENZYMES OF WHITE RATS

John M. Woodward, Ph.D.*

Severe metabolic disturbances are known to occur during infection with many pathogenic organisms including extensive alterations in enzyme levels in tissues and sera of infected hosts. Gilfillan et al.¹ reported marked changes in several enzymes of the tricarboxylic acid cycle in chicks during infection with Salmonella pullorum and Woodward et al.² showed that infection with Pasteurella tularensis stimulated a depletion of free amino acids in the blood of white rats. The fact that various pathological conditions alter enzyme levels in the blood suggested the possibility that changes in enzymatic activity might be of value clinically as indicators of specific infection or organic abnormalities in tissues or organs. For example, Hsieh and Blumenthal³ reported elevated serum lactate dehydrogenase (LDH) activity in myocardial infarction and after investigation concluded that elevated LDH was a good index of this type of heart damage.

In recent years, many enzymes have been found in measurable and predictable concentrations in the sera of numerous laboratory animals and in normal and infected individuals. This has prompted further study of changes in enzymatic activity as clinical tests for infection. Recently, Lust and Squibb⁴ observed changes occurring in serum alkaline phosphatase activity of chickens infected with the virus of Newcastle disease before overt illness was apparent. Hence, the possibility exists that alteration in serum enzymes prior to clinical manifestations may occur predictably in other infections, further enhancing the value of enzymatic tests as indicators of infection.

This investigation was undertaken in an effort to determine metabolic changes occurring in animal hosts prior to the appearance of frank clinical symptoms resulting from specific bacterial infection. A study was made of several enzymes present in the serum of white rats for evaluation of metabolic changes occurring as a result of infection with P. tularensis, Salmonella typhimurium, and exposure to the endotoxin of S. typhimurium.

Adult, white, male rats weighing 180-250 gm (Sprague-Dawley, Inc., Madison, Wisconsin) were employed throughout. The animals were maintained in air-conditioned quarters and provided with Purina Laboratory Chow and water ad libitum.

The highly virulent SmS4 strain of P. tularensis was employed in this investigation. The organism has a median lethal dose (LD₅₀) for the rat of approximately 3.5×10^3 organisms. A virulent strain of S. typhimurium was employed which, although not lethal for rats, caused severe illness of 2 days duration following administration of large numbers of viable cells.

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The low virulence Jap strain of P. tularensis was used for preparation of killed cell suspensions. The organisms were grown for 18-24 hr at 35°C (with shaking) in peptone broth.⁵⁷ Cultures were adjusted to contain 1% formaldehyde and allowed to stand overnight at room temperature. Cells were recovered by centrifugation, washed 3 times in physiological saline, re-suspended in physiological saline containing 0.1% formaldehyde and stored at 10°C.

A commercial preparation of S. typhimurium endotoxin (Bacto-Lipopolysaccharide W Salmonella typhimurium, Difco Laboratories, Detroit, Michigan) was used in studies of endointoxication.

Rats were injected via the intraperitoneal (IP) route with P. tularensis in concentrations averaging 3.5×10^9 , 3.5×10^3 , and 3.5×10^1 organisms per animal. The same procedure was employed with S. typhimurium for administration of cell concentrations averaging 2.5×10^9 and 2.5×10^3 organisms per rat. Serum enzyme activity was also studied following administration of killed cell suspensions of P. tularensis (Jap strain) at concentrations of approximately 3.5×10^9 cells per rat, and of S. typhimurium endotoxin averaging 7.5 mg per rat.

Noninjected animals and animals receiving sterile physiological saline IP were used as controls throughout the study.

At appropriate time intervals following injection, blood was obtained by cardiac puncture using a 5-ml syringe with a 20 gauge needle from all rats while under ether anesthesia. The blood was transferred to chilled centrifuge tubes and allowed to stand for 15 min. After clotting, the specimens were centrifuged at 7000 x g. for 15 min in a Sorvall refrigerated centrifuge (Model RC-2, Ivan Sorvall, Norwalk, Connecticut); sera was decanted and refrigerated or frozen until used. All hemolyzed serum samples were discarded. Pooled sera from 4-5 rats were employed for each enzyme determination.

Serum enzymes under investigation included aldolase, glutamate-oxalacetate transaminase (SGOT), isocitrate dehydrogenase (SICD), SLDH, and phosphohexose isomerase (SPHI). Determination of glutamate-pyruvate transaminase (SGPT), arginase, acid and alkaline phosphatases and ornithine carbamyl transferase activity also were conducted. In nearly all instances enzyme activity during infection followed a similar pattern. In addition, no significant changes were noted in the activity of leucine aminopeptidase, sorbitol dehydrogenase or cholinesterase.

The data obtained have shown that tularemia infection in rats is indeed associated with significant increases in the activity of specific serum enzymes and that the magnitude and time of these changes occur following infection is directly related to the size of the infecting dose (Figure 1). Administration of high concentrations of P. tularensis resulted in significant alterations of serum enzymes within 6-12 hr,

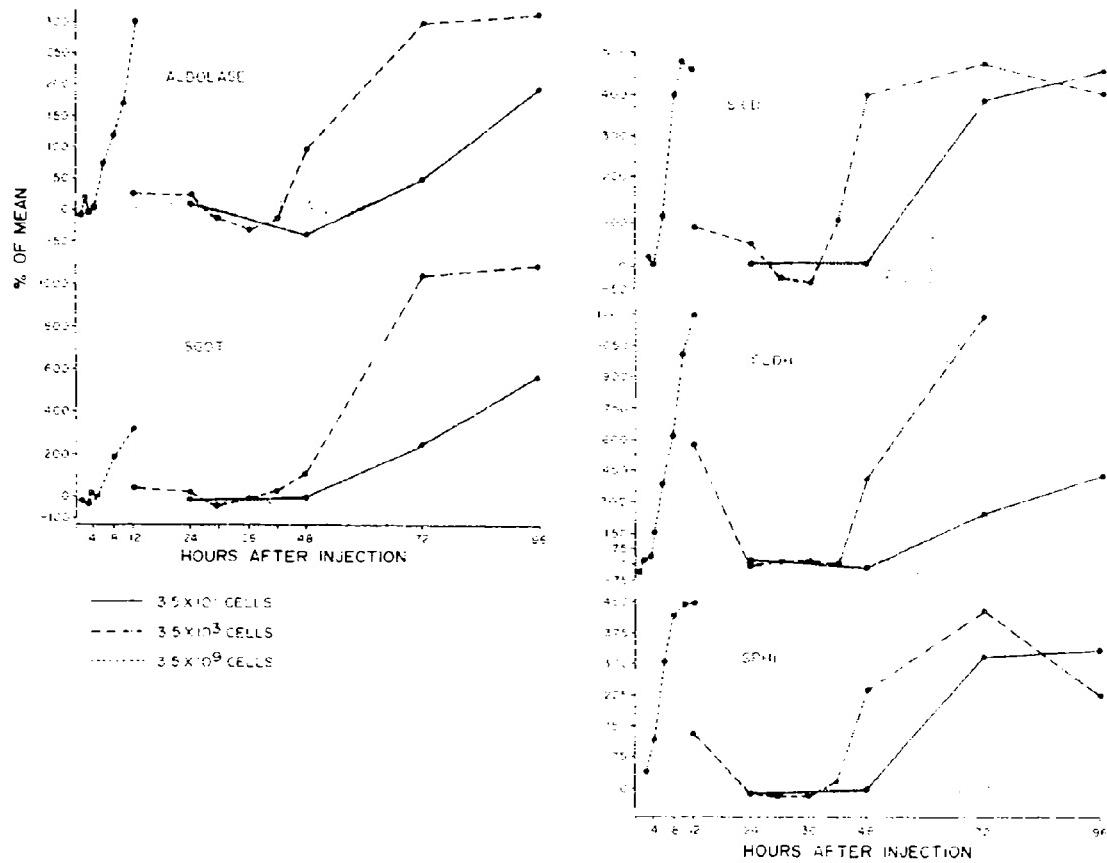


FIGURE I. SERUM ENZYME ACTIVITY FOLLOWING INFECTION
WITH VARIOUS CONCENTRATIONS OF
P. TULARENSIS. NORMAL RANGES ARE SHOWN
STIPPLED.

while similar changes following infection with progressively smaller dosages were not noted until after 42-72 hr. The rapid initial increase in activity after infection followed shortly by a return to normal values has not yet been explained. This phenomenon may represent a nonspecific reaction to the presence of foreign material in the peritoneal cavity, since similar results were obtained following the administration of high concentrations of nonviable P. tularensis to normal rats. Pronounced secondary increases, however, were correlated with progressive infection and extensive involvement of the liver. These increases in enzyme activity were routinely observed 6-24 hr prior to the appearance of overt symptoms.

Our observation of changes in enzymatic activity in tularemic rats are similar to those noted by Arean^{6/} in guinea pigs suffering from leptospiral infections in which elevation in activity corresponded to increased severity of infection and decreasing activity paralleled a return to normal health.

Further evaluation of enzymatic changes required the investigation of activity in response to another organism. Therefore, S. typhimurium which is less virulent was selected for study and the concentrations of organisms employed were similar to those used with P. tularensis in order to allow meaningful comparisons of the two types of infection. The administration of 2.5×10^9 cells of S. typhimurium stimulated increases in serum enzyme activity within 2-4 hr which far exceeded those noted during the same time interval during infection with the more virulent P. tularensis (Figure 2). Recognition of the specificity of these early and extensive increases in response to infection with S. typhimurium was not possible from this study. However a rapid and permanent return to normal activity at 24 hr indicated more effective host resistance than in tularemic infection, with suppression of further proliferation of the organism. Significant secondary elevations in activity were noted for SGOT, which were not correlated with a noticeable increase in severity of the infection. Similar findings were reported by Kumate et al.^{7/} in studies of patients with typhoid fever. It is conceivable that the systemic nature, rather than major hepatic involvement, of Salmonella infections is responsible for the irregularity of the enzyme alterations noted above. Administration of the smaller concentrations of S. typhimurium stimulated no visible symptoms in the rat and induced only slight increases in enzyme activity, indicating that the dosage was insufficient to establish progressive infection.

Despite the difference in virulence between the 2 organisms for the rat, the general phenomenon of increases in enzymatic activity during both infections suggests that these are nonspecific. Quantitative differences in enzyme activity were observed but were considered to be dependent upon the severity, rather than the specific nature of the infection. The results are consistent with those noted by Hauss and Leppelmann^{8/} who found identical patterns of serum and tissue enzymatic

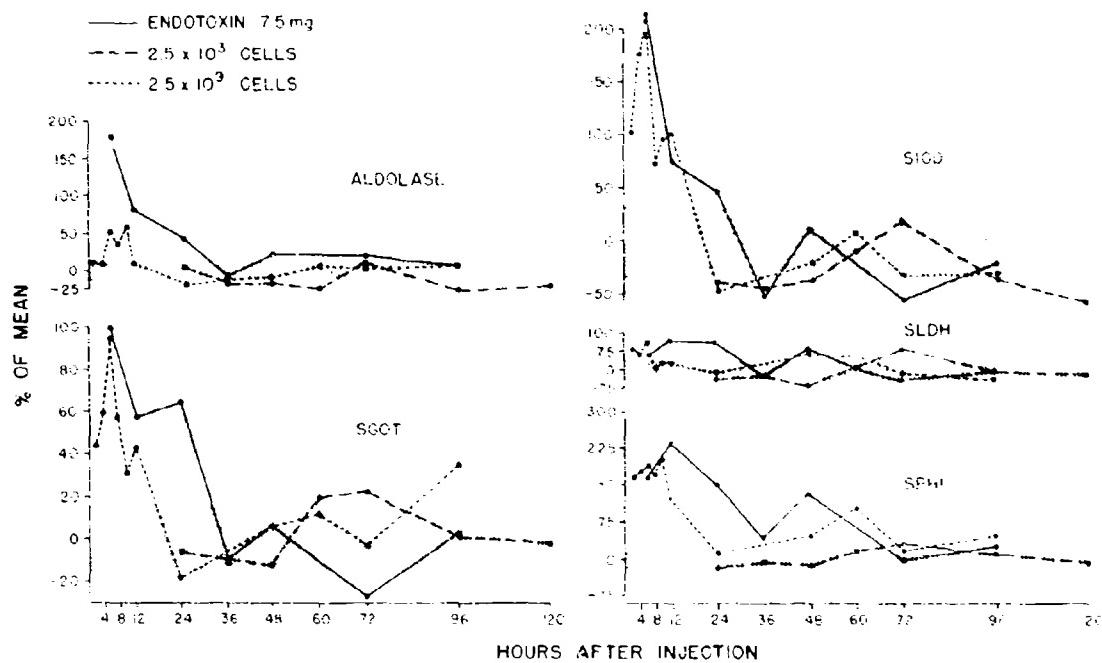


FIGURE 2. SERUM ENZYME ACTIVITY FOLLOWING INFECTION WITH VARIOUS CONCENTRATIONS OF *S. TYPHIMURIUM* AND INTOXICATION WITH ITS ENDOTOXIN. NORMAL RANGES ARE SHOWN STIPPLED.

activity in rats infected with pasteurellosis, paratyphoid B fever and tuberculosis and toxemia following administration of diphtheria toxin. Similarly, Saito and Suter⁹ found that alterations in activity of rat serum enzymes following infection with an attenuated strain of tubercle bacillus were duplicated by the action of a variety of agents such as polyvinyl pyrrolidine (periston).

Numerous studies relating the endotoxins of gram negative bacteria to antagonistic effects on host carbohydrate metabolism,^{10,11} nitrogen excretion,¹² and others raised questions concerning the extent of endotoxic involvement in serum enzyme changes. Recognition of the endotoxic properties of both P. tularensis¹³ and S. typhimurium prompted investigation with the latter's lipopolysaccharide. The fact that injection of rats with sterile physiological saline induced no change of serum enzyme activity indicated that alterations following the administration of endotoxin were due to specific action of the toxin. Subsequent observations of early increases in enzyme activity followed by the permanent return to normal values at approximately 30 hr are in close accord with the reports of other investigators. Kumate et al.⁷ administered 10 mg of S. typhimurium endotoxin to individual rats and noted a pronounced increase in serum arginase shortly thereafter with progressive decrease to normal values after 18 hr. Similar findings are also reported by Konttinen et al.¹⁴ in studies of SGOT, SLDH, and α -hydroxybutyrate dehydrogenase following intravenous injection of rabbits with endotoxin derived from Escherichia coli. Correlation of endotoxic shock with increases in serum activity is easily explained since endointoxication induces a circulatory imbalance resulting in reduced blood flow and release of catecholamines, followed by increased cellular permeability.

The magnitude and time of increase seen in the serum enzymes following administration of S. typhimurium endotoxin were fairly similar to those observed during infection with the parent organism indicating the possibility that endotoxic shock occurring as a result of infection with this organism is largely responsible for the changes noted. However, it was not possible to correlate the concentration of endotoxin with that released during infection with S. typhimurium. Furthermore, the site of action of endotoxin administered IP may be different from that of endotoxin produced during the course of infection. This was emphasized by Hill et al.¹⁵ who obtained an inbred strain of mice highly resistant to S. typhimurium endotoxin, yet demonstrated the susceptibility of these animals to infection with the same organism.

It is probable that no single factor is responsible for all aspects of serum enzyme alterations in response to pathological conditions such as infection or endotoxin administration. In addition to tissue necrosis, changes in enzymatic activity in the absence of cell destruction or cell death, have been reported by Hauss and Leppelmann,⁸ and have prompted further investigation. In general, any agent which alters cellular permeability, impairs the rate of clearing enzymes from serum, or alters

cellular metabolism may be responsible for alterations in serum enzyme activity.^{16/} The fact that cellular metabolism and permeability are modified by infection with P. tularensis^{17/} and by administration of endotoxin,^{14/} respectively, support the findings of this study. However, many aspects of this problem remain unexplained as evidenced by current investigation of mechanisms responsible for enzymatic changes.^{4/}

SUMMARY

Infection of white rats with P. tularensis and S. typhimurium and exposure to the endotoxin of S. typhimurium stimulated significant changes in various serum enzymes including aldolase, SLDH, SPHI, SICD, SGPT, SGOT, ornithine carbamyl transferase, arginase, acid phosphatase, and alkaline phosphatase. The rates of increase in enzymatic activity were directly related to the size of infecting dose, the type of infective agent employed, and to the severity and focus of infection. Tularemia infection stimulated excessive changes in enzyme activity while salmonellosis and endotoxication elicited less pronounced alterations which were of relatively short duration. The dissimilar changes observed in serum enzymes following exposure to these agents reflect the severe liver damage and extensive systemic involvement noted in tularemia as opposed to the more localized and less intensive tissue damage occurring during salmonellosis and endointoxication. Alterations in activity of serum enzymes frequently were demonstrated prior to the appearance of overt clinical symptoms of infection, suggesting the possibility of employing these procedures for early detection of infectious disease.

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DISCUSSION

DR. ATKINS: Did you do any studies to determine whether your latex was free of contaminating endotoxin? A number of workers have shown that latex particles may be contaminated, and one has to use precautions by heating at high temperatures for long periods in order to inactivate contaminating endotoxins. Some batches of latex particles prepared by Difco are contaminated with endotoxin. It is possible that phagocytosis of contaminated latex particles led to a release of enzymes from the leukocytes.

DR. JOHN WOODWARD: When we were studying hydrolase activity of peritoneal leukocytes the administration of latex didn't change hydrolase activity one way or the other.

DR. LEVY: A number of years ago Arthur Shade and I were studying enzyme levels in cases of influenza during an epidemic and we found rises in a number of glycolytic enzymes. The rises were real and associated with the disease. But confusing the picture tremendously was the fact that the method of collection of the serum and the method of the removal of the cellular elements greatly altered the picture. If the blood was allowed to sit around for any length of time, vast quantities of glycolytic enzymes leaked out of cellular elements. The method of centrifugation affected this as well, so the technique of handling these preparations must be uniform.

DR. JOHN WOODWARD: We attempted to remove the serum just as rapidly as possible. The temporary rise with endotoxin and then the subsequent fall might be related to the fact that endotoxin may lead to a release of proteins from cells. As you may know, endotoxin causes release from lymphocytes (either circulating or in the spleen) of the protein, "interferon." Perhaps the rise, fall, and then the subsequent rise is related to the concentration of endotoxin; some endotoxin may go in with the inoculum; it disappears; and, then, as organisms start to proliferate more fresh endotoxin appears to cause a subsequent release again of proteins from the cells. We use washed cells. I don't know whether this would eliminate much endotoxin or not; but the cell suspension contained 3,500 cells. I don't know how much endotoxin might be contained in this number, but it might serve to explain the early rise. Certainly we got an earlier increase and a more pronounced one following administration of S. typhimurium than with P. tularensis using high concentrations of cells. Even though ultimately the infection associated with this organism seems to be earlier (from 2-4 hr after administration) and more extensive.

DR. GRAY: We too have recent findings on the leukocytic enzymes. We have compared the peripheral leukocytes and the peritoneal leukocytes, studying their general esterase activity and we've found no difference. But in doing a zymogram of the enzymes from rat leukocyte homogenates, we find a marked difference in the molecular form. We found the same also with LDH. In rabbits, as early as 4 hr after infection with pneumococcus, not only has the LDH activity gone up but we have detected the appearance

of an additional form of the LDH. Normally, the rabbit (I use the word, normally, very carefully) leukocytes show 4 LDH bands; within 4 hr after the infection and before any fever there appears an additional, fast, number 2 band. We found a very marked appearance of the same band also in peripheral leukocytes of a patient with a Pseudomonas infection. In 2 patients who recovered from a Pseudomonas infection, this band had disappeared.

DR. KNIGHT: In work with volunteers, we had occasion to test the SGPT and SGOT values before and during the course of infection with rhino virus, Coxsackie 821 and adenoviruses 4 and 7. We were provoked into this by the occurrence of a case of infectious hepatitis in one volunteer. The result of our study was that normal volunteers not infrequently have slight elevations of SGPT and SGOT values before you inoculate them with anything and that these values don't appear to do very much in the course of infection with these 3 agents. I wouldn't say that there was absolutely no effect from inoculation and illness with these viruses but it is not very great. With the normal background variability of our enzyme determinations, we were not satisfied that there was a positive effect.

SERIAL CHANGES IN CELLULAR ENZYMES

Morton I. Rapoport, M.D.*

Last year preliminary data were presented^{1/} to indicate that induced pneumococcal infection in experimental animals could cause marked alterations in hepatic enzyme synthesis and hepatic protein metabolism, in general well before the onset of clinical symptoms. A specific identifiable protein selected for detailed study was the liver enzyme, tryptophan pyrolase (TP).

This enzyme was selected as a model for a number of reasons. (1) It had been shown by Berry and Smythe^{2/} that alterations of this enzyme seemed critical to survival of animals with gram negative infection; (2) the enzyme itself has a very rapid turnover rate and therefore is likely to manifest changes very early in the course of infection; and (3) this enzyme is rate-limiting in the vital pathway in which nicotinamide is produced, a product utilized in a large number of biologic oxidations.

It seems in order to review some of the data presented last year^{1/} with the more recent extension of this work and then present very recent studies performed in man.

During our initial investigations we observed that TP activity of liver homogenates varied relative to the time of day that the mice were sacrificed. It became apparent that we were dealing with an enzyme with a diurnal rhythm. This had not been reported by other investigators; therefore, mechanistic studies of this phenomena were performed. Very briefly measurements of enzyme activity throughout the 24-hr clock indicated that peak activity occurred in mice at approximately 2100 hours (solid line, Figure 1). Interestingly enough maximum enzyme activity occurred 4-5 hr after maximal corticosterone levels. Knox and Auerbach^{3/} had shown earlier that pharmacologic doses of adrenal glucocorticoids could induce TP in experimental animals with maximal induction also occurring 4-5 hr after steroid administration. Suspecting that the diurnal rhythmicity of TP activity was entrained in the adrenal cycle measurements were made of TP in adrenalectomized mice (dashed line).

The absence of the adrenal gland caused a loss of normal rhythmicity of TP. Observations indicated that studies of this enzyme during the course of infection would require appropriately matched controls throughout the period of investigation. With this knowledge measurements of TP activity were made in mice sacrificed serially after induced pneumococcal infection. A standard inoculum of 50-100, Type 1-A5, Diplococcus pneumoniae were injected subcutaneously into mice weighing 25-30 gm. The infection was always lethal and generally the mice died < 64-72 hr postinoculation.

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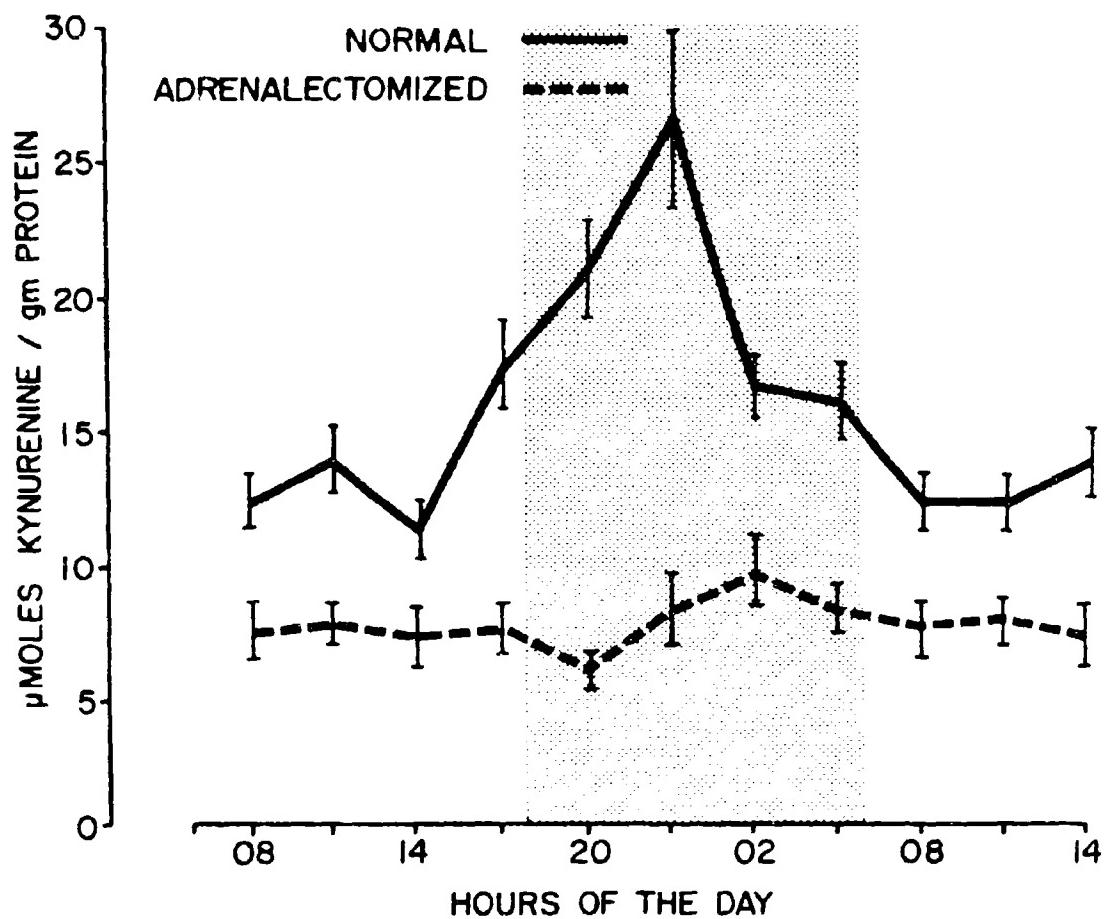


FIGURE I. TP ACTIVITY IN NORMAL AND ADRENALECTOMIZED MICE.

A significant increase in enzyme activity occurred within 4-6 hr and reached more than twice normal by 12 hr postinoculation (solid line, Figure 2). The increase in enzyme activity was not sustained however since values fell to control levels within 24-36 hr. The role of the adrenal gland secretions was then investigated by studying adrenalectomized mice in a similar fashion. Pneumococcal infection in these mice was more fulminate in that death generally occurred in < 40 hr. The previously observed early increase in enzyme activity was not present in these mice (without-replacement line, Figure 2) furthermore simple physiologic replacement of cortisol was not sufficient to restore normal responsiveness of TP (dashed line). These studies indicated to us that adrenal glucocorticoids played a primary role in the induction of TP during infection and furthermore that this role was more than permissive in that exogenous steroid replacement did not restore TP responsiveness to normal.

The results of these studies in which a single liver enzyme was examined during the course of infection seemed to us analogous in a sense with Dr. Lust's investigations of total hepatic protein synthesis during pneumococcal infection.⁴⁷

A brief review of these studies follows along with how they have been extended. By means of a standard technique in which labeled-amino acids are incubated *in vivo* for 50-min pulses, the rate of incorporation of amino acid into protein may be determined. Isolation of the microsomal fraction assures that one is measuring primarily new protein formation. Mice were infected in the manner described previously⁴⁷ and C¹⁴-leucine was injected approximately 50 min prior to sacrifice. Livers were removed at the time of sacrifice and pooled in groups of 4. Microsomes were isolated; radioactivity of protein was determined with a liquid scintillation detector. Serial measurements of C¹⁴-leucine incorporation into hepatic microsomes during the course of the infection are shown in Figure 3. Within 12 hr postinoculation a measurable increase in C¹⁴-leucine incorporation into protein occurs (solid line). This increased anabolic rate is progressive until about 40 hr postinoculation when a relative decrease occurs although values remain higher than control. A second series of experiments was then performed in mice that had been adrenalectomized 1 week prior to study. These studies indicated that the infection-related increased incorporation of C¹⁴-leucine was not present in adrenalectomized animals. Furthermore simple physiologic replacement of cortisol was not effective in restoring the normal response.

It was apparent that a similarity existed between TP activity and total hepatic protein synthesis during infection, both parameters exhibited nonsustained increases early in infection and both required an intact pituitary adrenal axis for these changes to occur.

The fact that the changes did not correspond exactly in timing is not difficult to reconcile. Although TP is a protein with an extremely rapid turnover rate, T_½ probably < 2 hr, it represents an infinitely

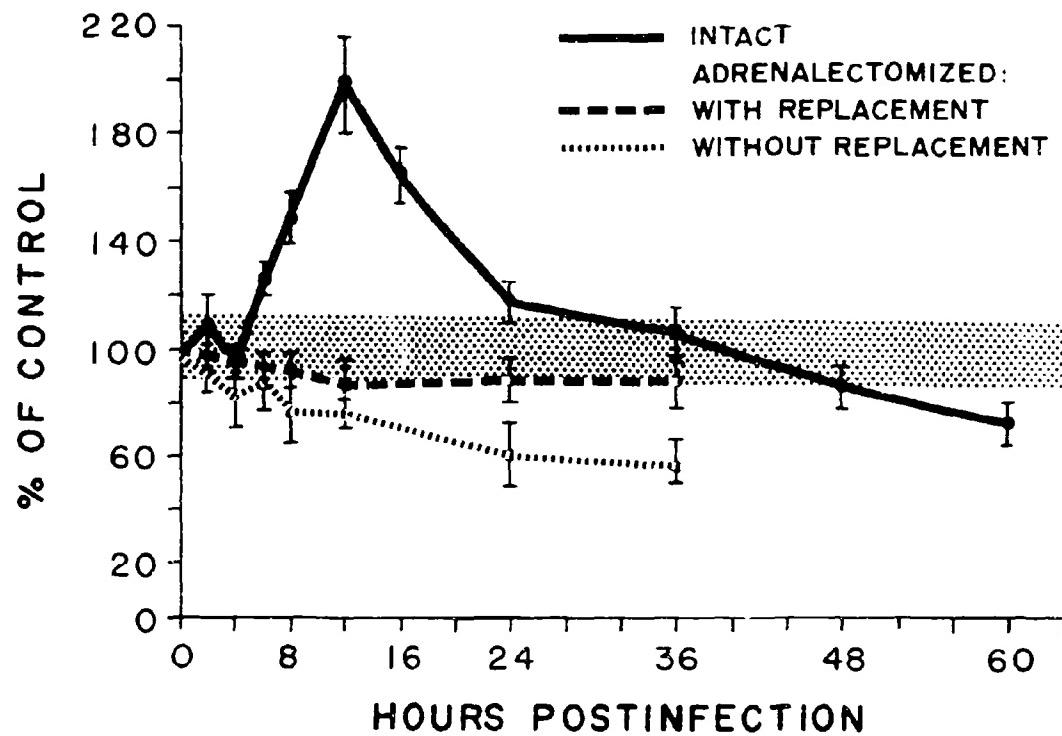


FIGURE 2. TP ACTIVITY IN EXPERIMENTAL PNEUMOCOCCAL INFECTION IN NORMAL MICE AND IN ADRENALECTOMIZED MICE WITH AND WITHOUT REPLACEMENT THERAPY.

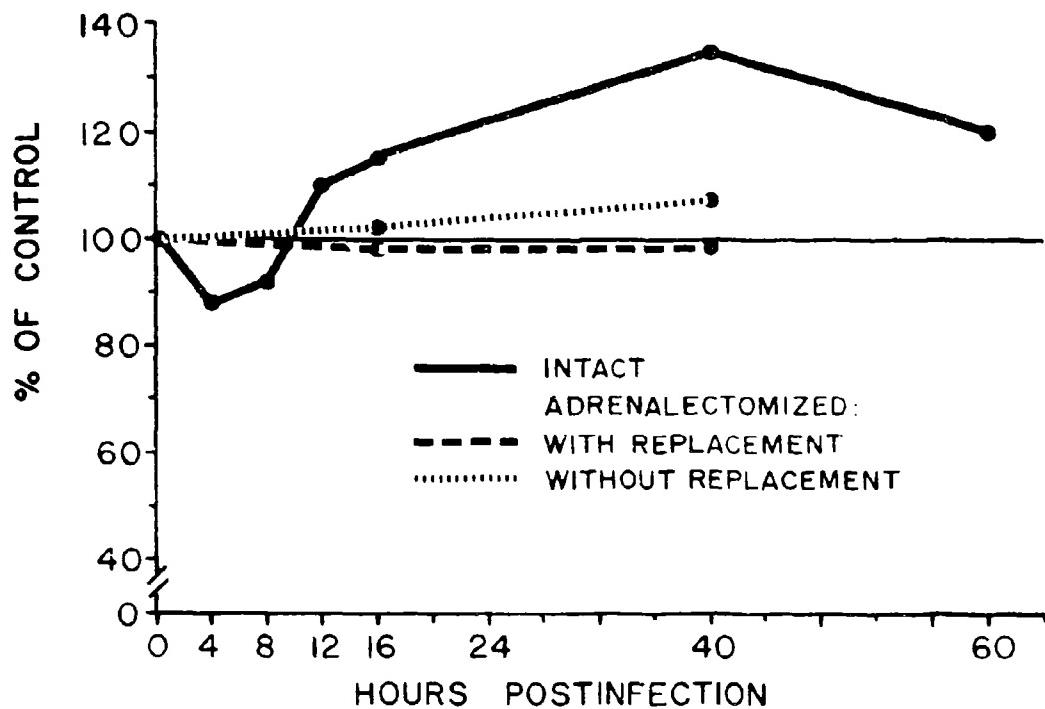


FIGURE 3. C^{14} - LEUCINE INCORPORATION INTO LIVER MICROSONES OF MICE.

small quantity of protein within the liver. In contrast, measurements of total protein anabolism within the hepatic microsomes reflect a broad variety of enzymes and other proteins, most of which have turnover times substantially longer than TP. Nevertheless it seemed justified to pursue the TP model in other infections for the reasons given at the outset.

With this thought in mind we examined a viral infection which also produced marked changes in hepatic protein metabolism. Figure 4 shows studies done by Lust^{4/} which showed that infection of mice with Trinidad strain Venezuelan equine encephalitis (VEE) virus, produced an initial decrease in hepatic protein biosynthesis followed by a progressive increase which seemed to parallel virus production (solid line). Therefore the augmented protein biosynthesis was interpreted as a function of viral protein rather than host protein. Further support for this interpretation was the demonstration of a new RNA polymerase in virus infected cells, not present in normal cells.

It was of some interest to examine the response of a specific host protein during this period of viral protein production. Also shown in Figure 4 are studies in which TP (dashed line) was measured. Initially a very transient increase in enzyme activity occurred followed by a fall which became progressive during the course of infection. Terminally enzyme activity in VEE-infected mice was < 1/3 that of controls. From these data it was concluded that concomitant with the augmented C¹⁴-leucine incorporation into what was apparently viral protein there was a reduction, or at least partial inhibition, of normal host protein synthesis.

Having established the fact that specific infection may produce in experimental animals changes in liver protein synthesis early in the course of illness, it seemed reasonable to define the existence or absence of these changes in man. The limiting factor in obtaining this information was the inaccessibility of human liver. It was apparent that indirect means of studying human hepatic protein synthesis and amino acid metabolism in general, during infection would be required.

At about that time in our thinking Altman and Greengard^{5/} reported measuring TP activity in liver biopsies obtained from patients given tryptophan loads. They showed that urinary excretion of the immediate product, kynurenine, was directly related in magnitude with the activity of TP in the liver homogenates obtained by biopsy.

However, the laborious and time consuming techniques required for measurement of kynurenine and all of the tryptophan metabolites did not lend themselves to large scale studies.

A chart of tryptophan pathways illustrates several points (Figure 5). First the entire lower portion represents products of the nicotinic acid (or niacin) pathway; TP is active here. Serotonin and indican pathways

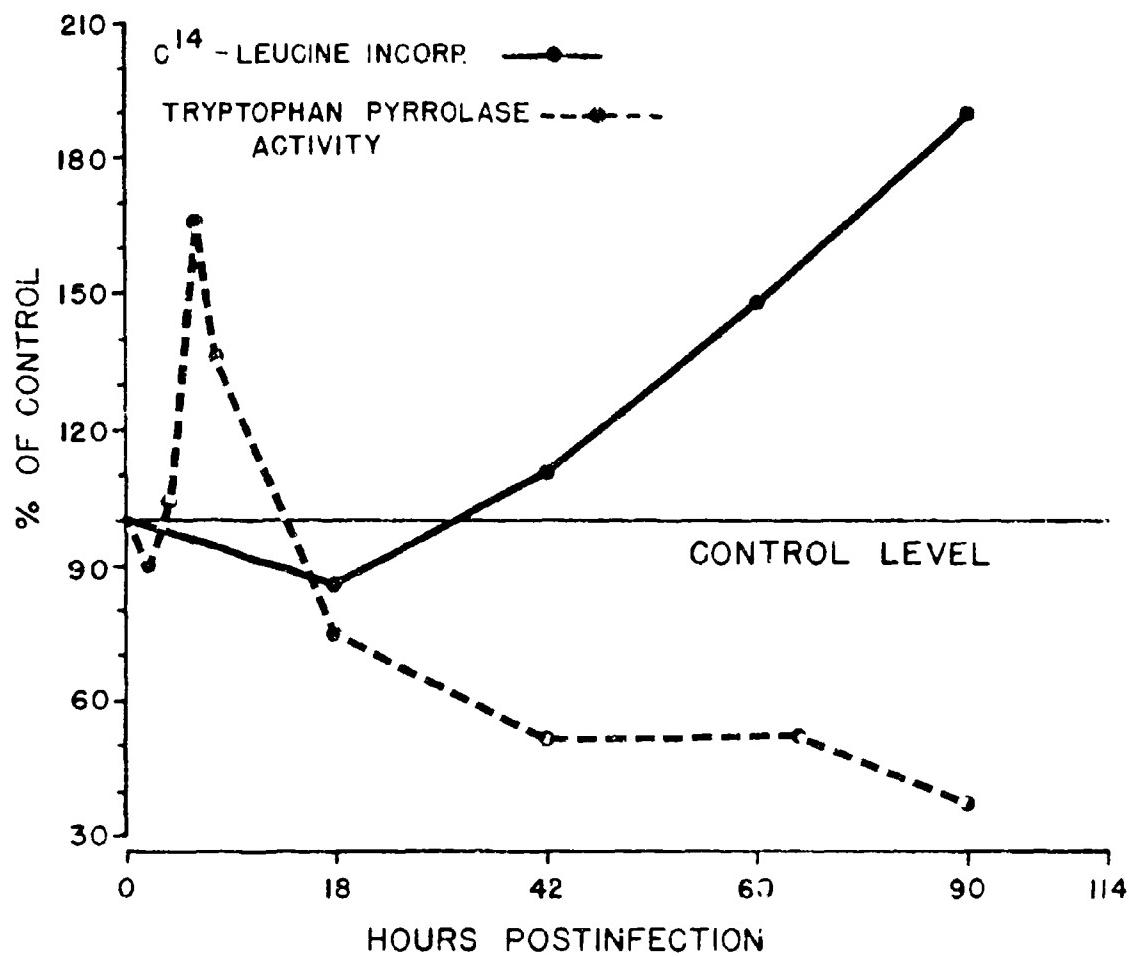


FIGURE 4. ALTERATIONS OF PROTEIN METABOLISM DURING VEE INFECTION IN MICE.

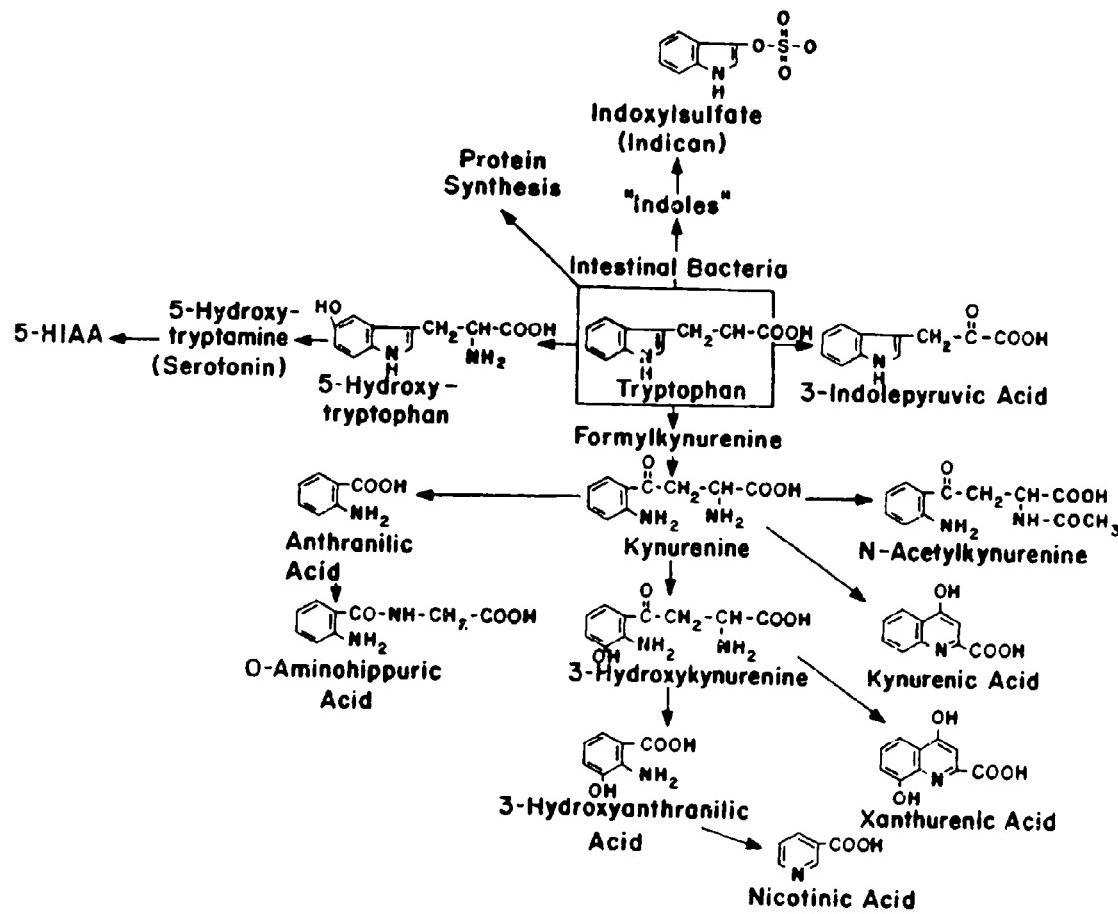


FIGURE 5. TRYPTOPHAN PATHWAYS.

are also shown. The point to be made with the structural formulae is that most of the metabolites of the niacin pathway are aromatic amines. Thus they may be diazotized, i.e., they will react with NaNO_2 to produce compounds which may be measured by colorimetric methods; this is called the diazotization procedure. The urinary diazo reaction was the subject of much early research in infectious illness by Ehrlich,^{6/} and in fact was called the Ehrlich diazo reaction. However, it was not until 1931 when a group of Japanese workers^{7/} showed that the quantity of diazotizable aminos in the urine was a function of tryptophan breakdown, and specifically of the niacin pathway.

These observations seemed especially relevant to the report of Altman and Greengard^{5/} in which metabolites of the niacin pathway varied directly with TP activity after tryptophan loading. It appeared feasible to study specifically this pathway and perhaps other aspects of protein metabolism under circumstances of amino acid loading during infectious illness.

Utilizing an automated technique for diazotization of urine it has been possible to study a large number of volunteer subjects given tryptophan loading tests under various circumstances. The results of these studies follow.

The first series of experiments was designed to confirm the fact that measurements by our method of urinary diazotizable amines were a reflection of tryptophan metabolism. A modified dose-response study was performed on a group of subjects. Figure 6 shows the curves for tryptophan loads varying from 1-4 gm, the dose range utilized by most investigators, plotted against urinary excretion of diazotizable amines. A direct linear relationship which is highly significant is evident from these studies.

A second series of loading tests was performed to document any change in tryptophan metabolism as a function of the time of day. Since TP had a circadian rhythm in mice which appeared to be entrained within the adrenocortical cycle we therefore questioned whether a diurnal rhythm for this enzyme might also exist in man. The enzyme being rate-limiting in the niacin pathway it would seem reasonable to expect that tryptophan metabolized by this pathway might vary relative to the time the amino acid was ingested.

Therefore a standard 2-gm tryptophan tolerance test was performed in 51 men at different times during the 24-hr clock. Urine was collected for 6-hr periods and diazotizable amines were measured; results are shown in Figure 7. The horizontal scale depicts time of tryptophan administration and the vertical scale represents the measured quantity of diazotizable amine. The data was expressed relative to creatinine excretion in order to account for the influence of diurnal variation of glomerular filtration. The mean excretion of diazotizable amines was almost 3-fold greater at 0600 and 0900 than at 1800 and 2100 hours, $p < 0.001$. These data have been interpreted to suggest the existence of a diurnal rhythmicity for TP in man which is entrained within the adrenal cycle.

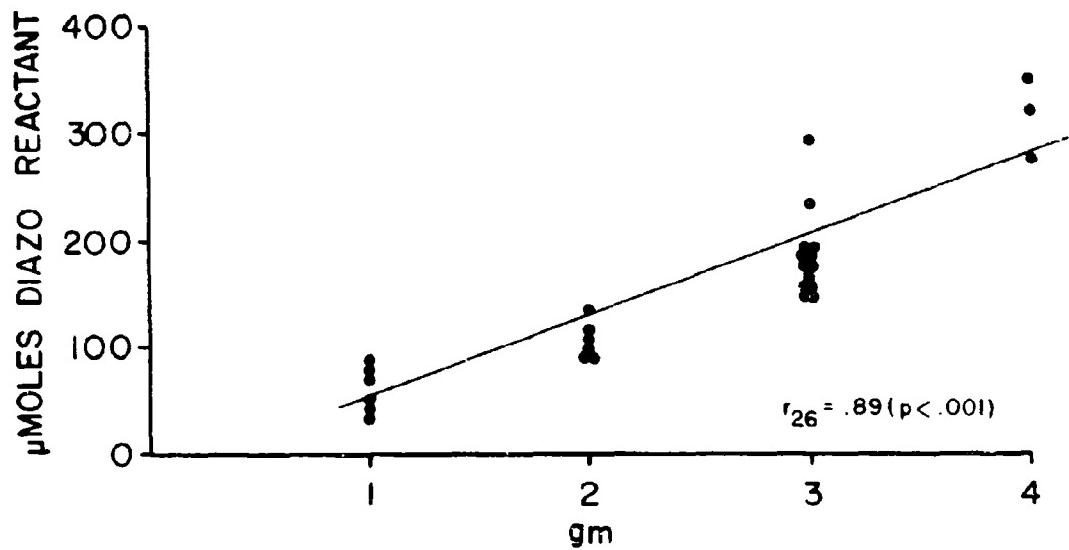


FIGURE 6. DOSE-RESPONSE RELATIONSHIP FOR TRYPTOPHAN TOLERANCE IN MAN.

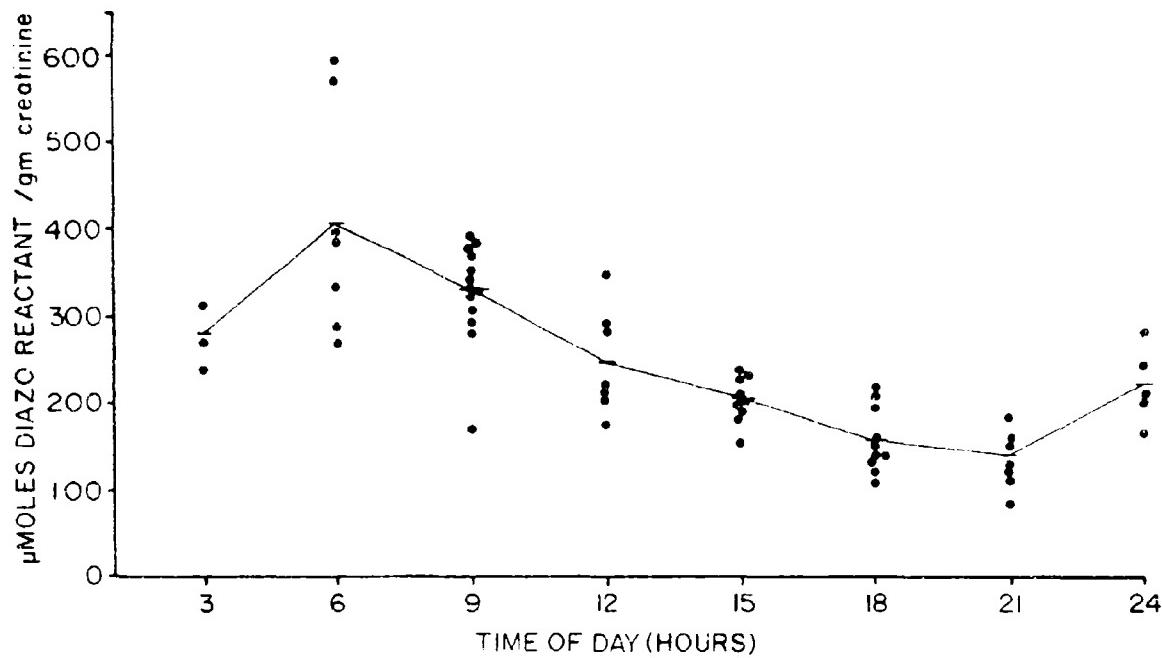


FIGURE 7. DIURNAL RHYTHMICITY FOR TRYPTOPHAN TOLERANCE IN MAN.

In order to confirm further that rhythmicity does exist, we utilized the somewhat laborious elution column chromatographic techniques on those tests done at the peak and nadir of the curve, 0900 and 2100 hours, respectively. By these methods the metabolites of the niacin pathway could be measured individually rather than in combination as was done by our automated procedure. Shown in Table I are the results of these studies and it is apparent that all tryptophan metabolites measured at 0900 hours with the exception of indican exceeded the 2100-hour values. While indican

TABLE I. URINARY EXCRETION OF TRYPTOPHAN METABOLITES AFTER TRYPTOPHAN LOAD (2 gm) AT 0900 AND 2100 HOURS

METABOLITES	EXCRETION	
	0900	2100
Indican	135 ± 26	209 ± 34
Kynurenine (includes acetylkynurenine)	79 ± 15	28 ± 5
O-amino hippuric acid	16 ± 2	10 ± 2
3-hydroxykynurenine	28 ± 4	13 ± 3
Anthranilic acid (as glucuronide)	19 ± 3	12 ± 2
Kynurenic acid	96 ± 12	25 ± 6
Xanthurenic acid	42 ± 6	15 ± 4

is a tryptophan metabolite, it is not a metabolite of the niacin pathway; in fact, it is derived from tryptophan which is metabolized by intestinal bacteria. For this reason the divergence of indican from the pattern observed for the niacin pathway assumes some special significance. It would appear that indican excretion is reduced in the morning hours because of the preferential shunting of tryptophan through TP or the niacin pathway. It may be speculated further that utilization of this amino acid for protein synthesis might bear a similar temporal relationship, i.e., more tryptophan is available for protein formation in the evening hours than in the morning.

Several conclusions appear warranted from the data presented (1) TP appears to have a circadian rhythm in man; (2) tryptophan tolerance tests which have been utilized for a number of diagnostic reasons should be controlled relative to time of day, and (3) it seems reasonable to assume that an optimum time of day exists for the ingestion of this amino acid and perhaps for other amino acids. This knowledge may serve a very practical purpose in patients whose nitrogen intake must be critically regulated e.g., those patients with hepatic coma or chronic renal disease.

Thus, armed with the knowledge that we could inferentially study effectively in man a protein biosynthetic pathway with the use of amino acid tolerance tests, we began studies with the same techniques during infectious illness. Very recently we had the opportunity to study a group of human subjects with induced sandfly fever. A series of tryptophan tolerance tests were performed in 4 volunteers who had been infected with sandfly fever virus; test results of 3 of them, who developed clinical illness are shown in Figure 8. Fever hours, measured excretion of diazo amines by automated technique, and quantities of kynurenine measured by the elution technique are plotted. In two of the individuals shown, F.S. and C.N., there was a > 2-fold increase over control values in excretion of diazo amines on the day preceding fever. Consonant with this increase was an increase of > 3-fold in excretion of kynurenine. The third individual had no significant change until the day of maximum fever when a 4-fold increase above control was observed in both parameters. The non-infected control and the very mildly symptomatic infected subjects showed no significant changes in diazo reactions in any of the tests performed. From these very preliminary data it would seem that early in the course of sandfly fever infection there is an augmentation of the tryptophan niacin pathway in some patients. Also it seems likely that the mechanism underlying this augmentation may be an induction of the liver enzyme, TP. It is quite evident that additional tolerance tests in infected patients will be required to substantiate these speculations. Nevertheless we believe we have some information that allows us to reevaluate the early reports of Ehrlich and others relative to the diazo reaction during infection and in addition perhaps to comment on some of the broader aspects of host protein metabolism during infection.

According to Ehrlich a positive urinary diazo reaction was regularly present in "typhus abdominalis, typhus exanthematicus, and advanced phthisis."⁶ Furthermore it was observed in various pneumonias and seemed to correlate with the severity of the infection. Other than recommending the use of the reaction for diagnostic and prognostic purposes, Ehrlich was unable to define the mechanism for this reaction.

Our investigations suggest that the alterations in the urinary diazo reaction Ehrlich described over 75 years ago are a function of stimulation of the tryptophan niacin pathway and specifically the enzyme tryptophan pyrolase.

The importance of changes in TP and other tissue enzymes in host defense mechanisms is highly speculative; however, it is evident these changes do occur in some varieties of infection.

Whether the changes in various enzymes have any specific diagnostic or prognostic significance or whether these changes merely reflect widespread alterations in protein synthesis is not clear from our data. However, it would appear that techniques such as amino acid loading and the administration of tracer-labeled amino acids might be useful in defining in man early biochemical alterations occurring as a result of infection.

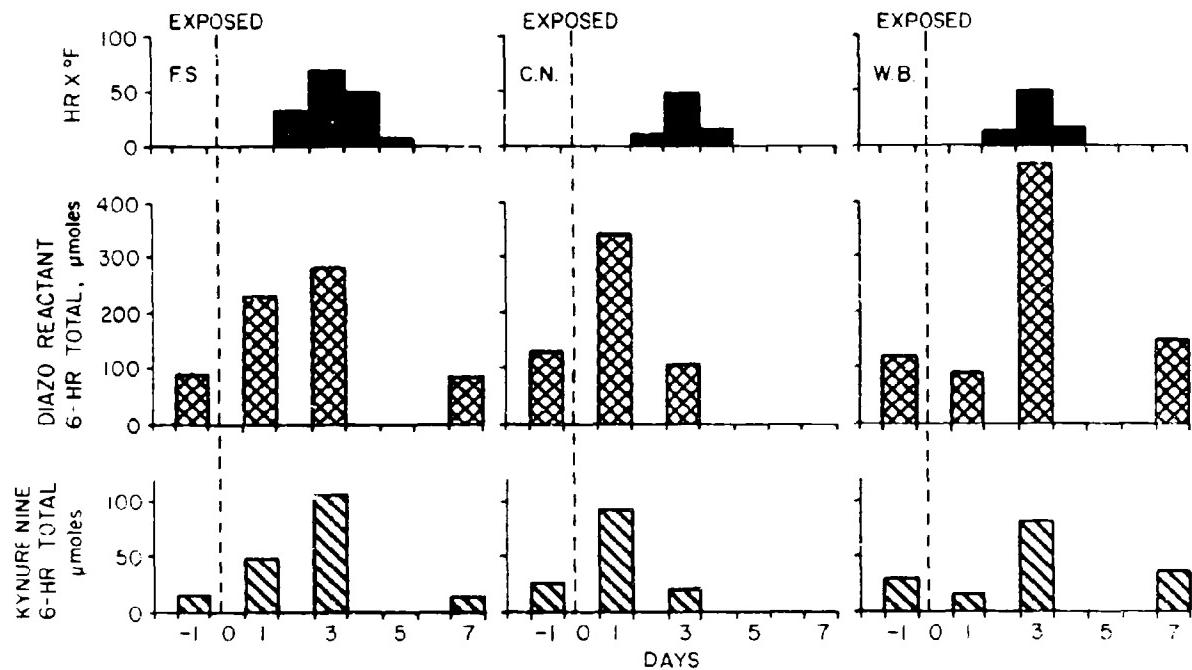


FIGURE 8. TRYPTOPHAN TOLERANCE TESTS IN MAN INFECTED WITH SANDFLY FEVER.

SUMMARY

Evidence is presented to indicate that induced pneumococcal infection in mice is capable of producing alterations in liver protein anabolism and a specific model liver enzyme, TP. These changes appear to parallel one another and seem mechanistically related in that intact pituitary gland function is a requirement for their occurrence. It is shown that the changes in enzyme synthesis and total hepatic protein anabolism occur very early in the course of infection, the implication being that these are biochemical events which precede the onset of clinical illness.

Studies designed to demonstrate the existence or absence of these changes in humans with infectious illness have been performed. It has been shown that the excretion of urinary diazotizable amines is directly related in magnitude to the quantity of tryptophan metabolized through the niacin pathway. Since TP is the rate-limiting enzyme for the niacin pathway it would appear that altered synthesis of this enzyme is the etiologic factor for variations in excretion of these amines. Prospective studies of volunteers with sandfly fever indicate that a significant increase in excretion of the amines may occur well in advance of fever and clinical illness.

It is suggested that at least one liver enzyme may be induced early in the course of human infection. The importance of this change in host defense mechanisms is highly theoretical. However, the vital role of nicotinamide in a large number of biologic oxidations indicates the need for additional knowledge of the tryptophan-nicotinamide pathway in infectious illness.

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DISCUSSION

DR. BESSMAN: You infer that the enzyme change is presumably, or at least, possibly, related to protein synthesis or occurs as part of the general picture of protein synthesis. If this is true, why did the TP drop during the period of apparent maximum protein synthesizing activity of the ribosomes?

DR. RAPOPORT: Well, I can't be sure, but I think that the data may allow some speculation. First, if TP is taken as a monitor (and I'm not sure that it should be), it is certainly more sensitive than C¹⁴-leucine incorporation into protein. Since TP is induced earlier than total protein synthesis it would appear that the enzyme reflects stimulatory influences more quickly. Similarly, it is conceivable that with its more rapid turnover rate, TP is also perhaps more sensitive to inhibitory influences. As you may remember late in the course of experimental infection there was a relative decrease in incorporation of C¹⁴-leucine into protein. I wouldn't care to speculate any further than that.

DR. LEVY: The question of interpreting the amino acid incorporation in the whole animal situation is, as you know, fraught with difficulty. It's bad enough when one deals with a tissue culture system where you can control or at least measure the free amino acid pool size. Can you really imply that you have increased protein synthesis because you have increased amino acid incorporation into protein? This is a real problem, and I wonder about the interpretation, for example, in that situation where you had increased amino acid incorporation into protein after VEE virus. The magnitude of increase in amino acid incorporation was quite large, and to attribute it to an increase in virus protein alone is questionable. The amount of virus protein formed would hardly be as much as your measured increase in the liver. Could this be a change in permeability, amino acid pool size, or something like that?

DR. RAPOPORT: Perhaps, but I would say two things in reference to that. First, we studied amino acid incorporation by hepatic microsomes, so presumably this would give us a better measure of total hepatic protein anabolism than such a measurement in the total liver homogenate. Second, in a small number of studies in VEE-infected adrenalectomized mice, the incorporation seems to parallel that of the intact mouse. Thus, we have ruled out the possibility that this increase is due solely to adrenal glucocorticoids. We have no information on the free amino acid pool size.

DR. MacLEOD: Concerning the correspondence between TP and total protein synthesis, one would not necessarily expect these to correspond, since the TP after all is "cortisone-inducible" and one cannot attribute such induction to all of the increased protein synthesis as evidenced by leucine incorporation. Is this correct?

DR. RAPOPORT: Yes, I do not believe that all of the components contributing to a net increase in hepatic protein anabolism during infection are necessarily cortisone-induced.

DISCUSSION OF SECTION I

Samuel Bessman, M.D.*

This is a very exciting thing, the investigation of relations between the organism and the host from the biochemical standpoint. The fact is that we have very little information at the present time. However, I recall the following statement "Give me a place to stand and I will construct a lever that will move the world." Now there are about 3,000 known proteins. This provides us with a great deal of work in the future if we wish to investigate each protein. I challenge any mind or even any computer to remember all these proteins and relate one to the other. So therefore, it might be a good idea to stick to a few likely models and attack these problems in a direct way.

Looking at protein synthesis is a very difficult problem, as was pointed out by Dr. Levy, because there are so many factors that enter into it. There are 3 major types of problems. First are problems of a technical nature. What is our standard of reference? What do we measure? It sounds very simple to measure the incorporation of amino acid into a protein. Well, the first pit-fall in the approach was recognized 15 or 20 years ago when studies were begun in nephrotic patients. In the nephrotic patient, serum protein is very low so one would immediately assume that nephrotics are loosing protein or that they can't make it. In attempting to measure protein synthesis, a tracer amount of S³⁵-labeled amino acid was administered to a patient or a group of patients. Such studies disclosed a remarkable thing. Studies of albumin showed that the rate of incorporation of S³⁵ amino acid into albumin was more than twice the rate of a normal individual. Now what does that mean? An initial interpretation might be, that a person with nephrosis makes protein faster. Yet he has only a fifth as much albumin as normal individuals. Then, if you begin to look at the problem a little closer you say, "Wait a minute! How did we measure the activity?" What was done to give the radioactive amino acid in a dose per weight of child, or per kilogram of patient, or synthesizing system (whatever we might want to call it). In fact, it was always given in a constant dose. The dose was put into a patient who made "X" molecules of protein which were labeled and could be calculated as the counts per gram of purified albumin if this was identified very carefully. Now, in another child with a lower total amount of serum albumin we get "X" molecules for the sick child, a number that is equal to 2 times the healthy one. But if there is only half as much total albumin in the whole body of the child who is sick, and he makes exactly the same number of protein molecules, then the child who is sick will appear to have twice as much radioactivity in his serum. This can be erroneously interpreted to mean that he makes albumin twice as fast. In reality he could be making it a third as fast and have one-sixth as much total albumin and he'd

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look like he's actually making it twice as fast. This problem of interpretation hasn't yet been resolved.

The next problem concerns the pools of host protein in the tissue, a problem touched upon by Dr. Rapoport. Within the tissue, tryptophan pyrolase may represent one pool and another protein may be another one. You can even measure the incorporation of radioactivity to each pool and still not know the difference. One can try to correlate incorporation of radioactivity with the size of the pool. Now this is a very difficult problem. For example, how do you measure total body albumin? You can say, "I assume it's all available by some sort of measurement," but this is not so easy. Instead of adding the total test dose to a large amount of amino acid in the organism we add it to a small amount of amino acid. Now when the synthesizing mechanism faces a dose which is diluted by a large amount of amino acid it makes a very mildly radioactive material. When it's faced with a very highly concentrated tracer dose, then it makes a very active protein. The same number of molecules may be present in the second instance, but they have more radioactive amino acids in them because they were taken from what we call a "hotter" pool.

Now, if we look at Dr. Squibb's data, we can come to a very interesting type of experiment that can be performed. In one of these situations of infection, the lysine dropped about to one-half and the aspartic acid increased to approximately twice that of the controls. Now suppose that we give a tracer dose of lysine to the infected individual. We will come to the conclusion that he's incorporating lysine very rapidly into protein, twice as fast as the controls. On the other hand if we gave a dose of aspartic acid, he would appear to be incorporating only half as fast as the control. So use lysine if you wish to come to a conclusion the patient is making protein twice as fast, or use aspartic acid for an answer half as fast.

Now, it even gets worse than this. We are involved in a similar problem, so I can talk about it with a sense of anguish. Dr. Rapoport took ribosomes, microsomes actually, and measured their incorporation of radioactivity. This sounds like a good thing because it avoids the large amino acid pool. But unfortunately there is still a rub. What is the pool of protein in the microsome itself against which you measure activity? If one took the same amount of protein and determined radioactivity per milligrams of microsomal protein it would sound pretty good. But is it? What about microsomes that are not making protein, or at least microsomes not directly concerned in terms of a one-for-one relation in their synthesis? Suppose that the protein pool in the microsome is smaller but a lot more microsomes are used. The microsomes might be little shriveled up things under these circumstances; so although it looks like the same number of protein molecules are being used, in reality there would be a lot more little factories to make proteins. Microsomes wouldn't have to shrink much to get a remarkable difference in apparent protein synthesis. Actually the difference reported here was somewhere around 30-40%, which is a nice

difference. But, nevertheless, if a loss of 30% per microsome of protein had occurred, one would appear to show the same kind of picture.

We should try to deal with the cell itself, or the DNA of the cell. Thus, the DNA of the cell (which is supposed to be a number that we can grasp) is the rock on which we'll stand. Is that a critical and exact quantity? Well, most of the time it seems to be, although I agree with Dr. Squibb that there are problems about that. We must depend upon some standard of reference in order to say that the cells of an organism are making more or less protein.

There is a similar problem in interpreting findings in muscular dystrophy. For many years it was assumed that protein synthesis was higher than normal in muscle in patients with muscular dystrophy. If you put all the published papers together, you begin to realize that the reason the protein synthesis is apparently higher is that there is less protein in muscles to start with. If you recalculate the data on the basis of protein synthesis per cell, you end up with the same, or less synthesis of protein in muscular dystrophy than there is in the normal individual.

These are a few of the smaller problems. Now there are larger problems. While changes are nice to report, I think it's always more interesting to find out what doesn't happen, because what doesn't happen may be a key to something we might call resistance. It's a key to something we might call specific organ effect. It's a key to mechanism. To me the most important thing about the enzyme studies is that there are enzymes which don't change. If we can find out what doesn't change, we might be able to do one of two things. We might be able to find out exactly where the trouble is, or better still, we might be able to find out how to resist the changes that do occur, changes that may be the causes of disease.

One of the most interesting things about these enzymes was Dr. Gray's comment on Dr. Woodward's paper concerning the de novo appearance of an isoenzyme of lactic dehydrogenase in the course of certain stress or disease situations. Now this is very exiting. What was done in the past was to take enzymes of the whole tissue, grind them up, mix them together and say, "Look at all the nice enzymes we've got here, more or less!" Well, we've been interested in what isoenzymes do. The question is, "How does a contracting muscle fiber tell an energy-producing mitochondria that it has just contracted, that it is again time to make energy?" Now, there must be communication. This communication is undoubtedly on a molecular scale. It's not a little distance! Miles! It's not microbial in size! There are literally millions and millions of molecules that have to elbow their way past each other in some way to communicate across a cell the size of a muscle fiber. Now, how does this occur? The muscle fiber contracts and ATP is converted to ADP. That's how energy is expended in the work that's done by the cell. Many phenomena of muscular dystrophy involve weakness; it involves the wearing out of the ability to perform work. Is weakness possibly an interference with the message getting through

the cell that says to the cell, "Forget it, don't bother about it." Maybe that is what a "bug" does. Maybe that is what a virus does. Maybe it is not all induction of particular enzymes. Maybe it is interference with particular isoenzymes.

So we ask these questions, and see if we can make a little model of one thing that we have evidence for. When the ATP goes to ADP it should then find a mitochondrion which we know converts ADP back to ATP. And that's called oxidized phosphorylation when the ADP hits the mitochondrion and the mitochondrion says "I understand." "I'll make another molecule of ATP," and immediately oxidation increases and the ADP is converted to ATP. It can't happen that way, because the contracted muscle fiber retains the ADP tighter than it retains the ATP. So the contracted muscle fiber is in a sense muscle-bound. It can't contract any more and it can't get rid of the ADP to go off and return as ATP. In fact, the ADP is blocking the ATP from getting back on. Now this sounds pretty bad. Now if you're going to design a new machine you would say, "Well, this is kind of foolish." Why can't it get off? But then you say, "Well, what about a fast muscle?" It would require that the ADP stays attached to the muscle and is reactivated in situ. The ADP can't go wandering off to find the energy someplace else. So, what happens is very simple. Everybody knows this. There's phosphocreatine which is a high energy compound, which in the presence of an enzyme, creatinin phosphokinase, transfers the phosphate to the ADP, making it ATP, and the muscle is ready to go again. And there's a very large pool of creatine phosphate in the muscle and it makes it contract; everybody knows this, so now after the contractions it's all gone.

But now how does the mitochondrion know that this has been going on? The muscle mitochondrion must now have a sensory mechanism. It must know that this pool of phosphocreatine is diminishing. Well, can do it in two ways? It can have something like a float valve which recognizes that the phosphocreatine is getting lower. This would be a small change in a big number. Or, it can have some signal mechanism for detecting the appearance of creatine. We know that normally there is no free creatine in muscle --- essentially none. It's all phosphocreatine in resting muscle. But the contracting muscle liberates a great deal of creatine (and this incidentally in some of the muscle diseases goes out in the urine. There must be a sensory mechanism in the mitochondria for creatine.) Well, 3 years ago, Klingenberg in Germany discovered that there were 2 isoenzymes of phosphocreatine kinase; one of them is soluble and the other one is in the mitochondria of muscle and brains, the 2 organs which are called upon immediately to move quickly and repeatedly. Only in these mitochondria are there such creatine phosphokinase isoenzymes. So we wanted to know if this was a signal receiver. We took mitochondria and added creatine to them and they "took off," increasing their oxidation, putting phosphate on the creatine just as if we had added ADP to liver mitochondria. In other words, here was a signal mechanism, here was a chemical process which was a cybernetic process which involved not only control but involved a buffer in which control was manifest from the action. Now, I'm telling

you all this for one simple reason; that we don't understand these isoenzymes. I'm delighted that people are now working with them in disease and in the function of cells, both in normal physiology and during pathology, to find out what their distribution has to do with the normal function. In model systems involving a bacterial or viral infection perhaps these connections between isoenzymes and particles, isoenzymes and surfaces may be disrupted in some way and we call this disease.

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SECTION II

CELLULAR NUCLEIC ACID CHANGES DURING INFECTION

MODERATOR: Dr. W. Barry Wood

DISCUSSANT: Dr. Hilton E. Levy

RIBONUCLEIC ACID METABOLISM DURING DISEASE

Captain J. Michael Kehoe, VC*

Previous studies from this laboratory have demonstrated time-dependent alterations in protein synthesis in various organs of mice experimentally infected with bacteria or viruses.^{1/} We have extended these studies, giving particular attention to the effect of the disease state on the utilization by intact mice of a specific RNA precursor.

Male, Charles River, CD-1 mice weighing 20-25 gm were used throughout. Bacterial infection was produced by the subcutaneous inoculation of 5-25 Diplococcus pneumoniae cells. Viral infection was produced by the intra-peritoneal (IP) inoculation of 3×10^3 median intracerebral lethal doses of stock Venezuelan equine encephalitis (VEE) virus (obtained from CPT A. Schwartz, Virus and Rickettsia Division, Fort Detrick). The mice were sacrificed in groups of 5 at various time intervals after inoculation. Two hours prior to sacrifice, the pyrimidine precursor, orotic acid-6-C¹⁴, was injected IP in a dose of 5 μ c per mouse. Liver, spleen, and kidney were removed and pooled for each group. After homogenization (Potter-Elvehjem), subcellular fractions were prepared by differential centrifugation (Figure 1). RNA was extracted from each fraction by a phenol method and quantitated spectrophotometrically. The radioactivity of RNA aliquots was determined by liquid scintillation counting; the specific activity of each RNA specimen was expressed as counts per min per optical density (at 260 μ m) unit. The specific activity of RNA preparations from infected animals was compared with values for uninfected controls and the results for the various stages of infection were expressed as per cent of control.

The rationale for the utilization of orotic acid in the study of RNA metabolism is presented in Figures 2-4. Figure 2 illustrates the relation of orotic acid to the pyrimidine base, uracil. The position of the C¹⁴ atom in the labeled compound used in these studies is indicated by the asterisk. Figure 3 shows biosynthetic and kinase steps involved in the production of uridine triphosphate and Figure 4 illustrates both cytidine triphosphate formation and a general scheme for RNA formation against a DNA template.

The results of studies on pneumococcal and VEE infections to date are summarized in Figures 5 and 6, respectively. The data for RNA isolated from the "soluble" fraction (postmicrosomal supernatant) are too spurious to interpret at this time and have not been included.

* U. S. Army Medical Unit.

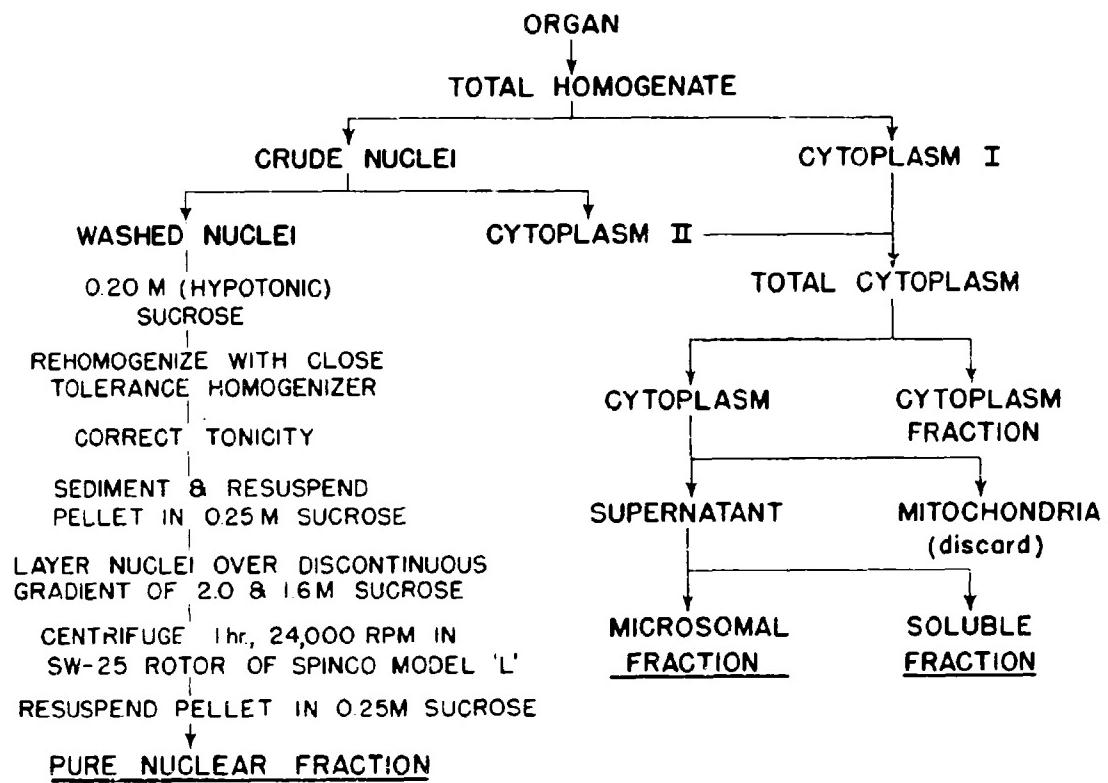


FIGURE I. PHYSICAL SEPARATION OF SUBCELLULAR FRACTIONS.

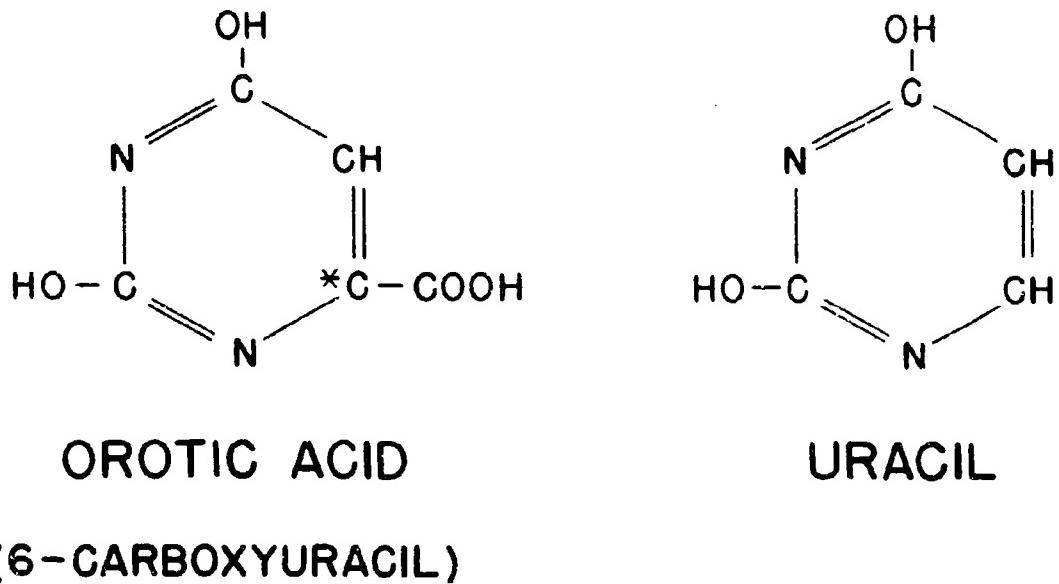


FIGURE 2. STRUCTURAL FORMULAE FOR OROTIC ACID
AND URACIL.*=LOCATION OF C¹⁴ ATOM.

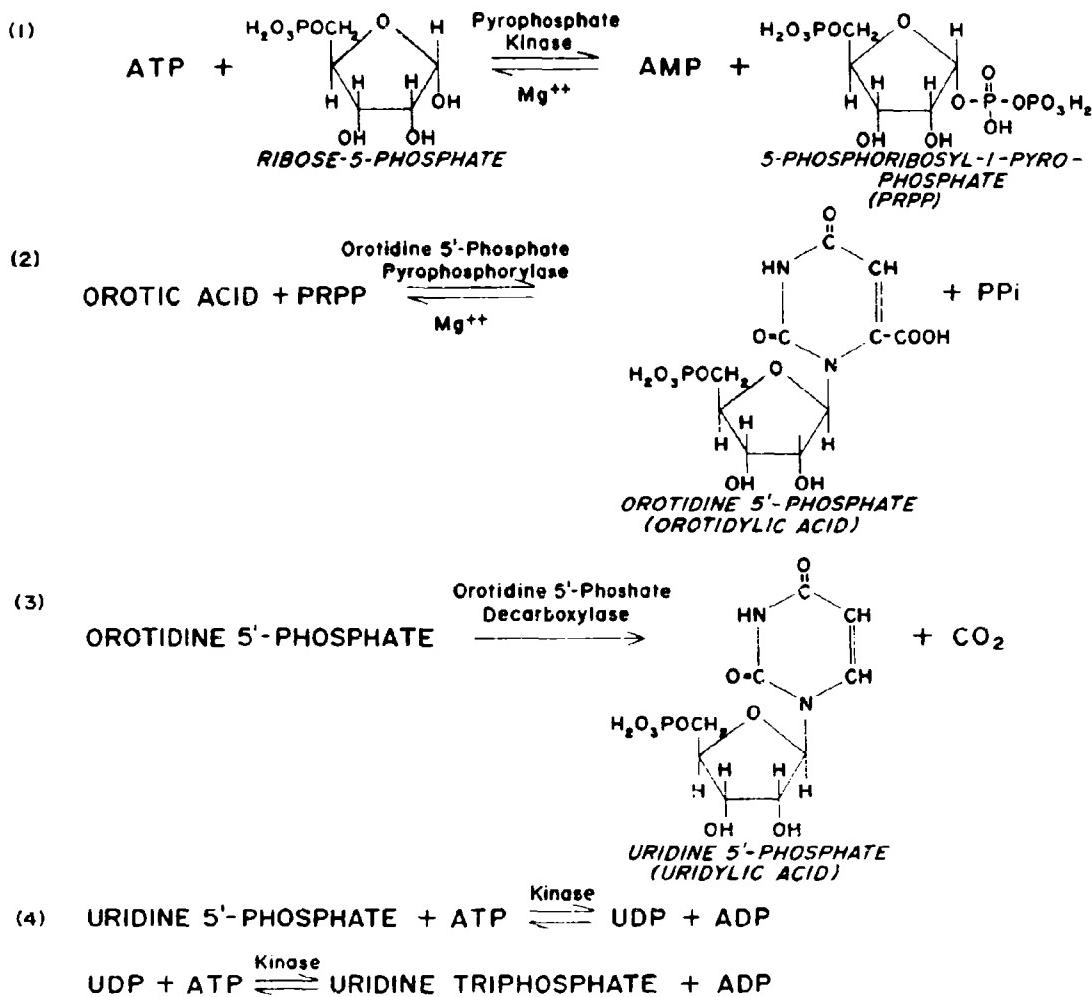


FIGURE 3. PYRIMIDINE NUCLEOTIDE SYNTHESIS.

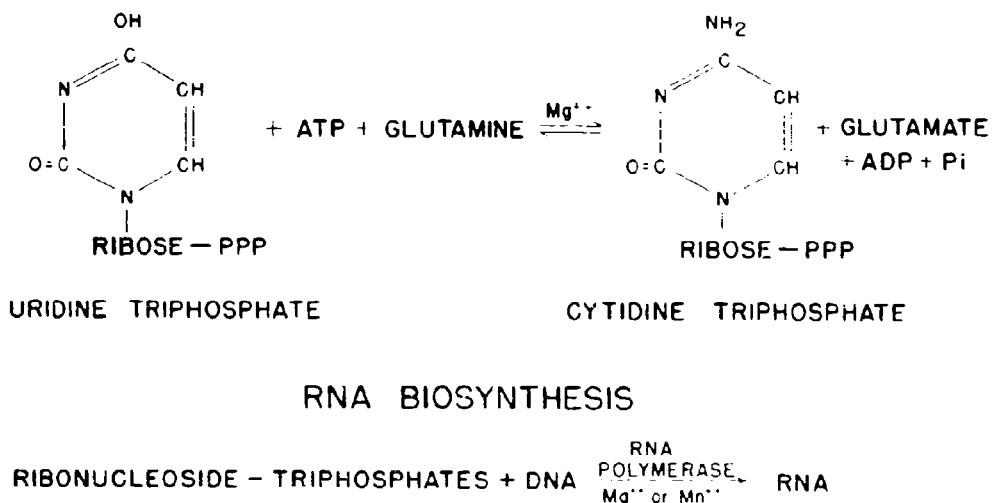


FIGURE 4. PYRIMIDINE TRIPHOSPHATE CONVERSION AND GENERAL SCHEME FOR RNA BIOSYNTHESIS.

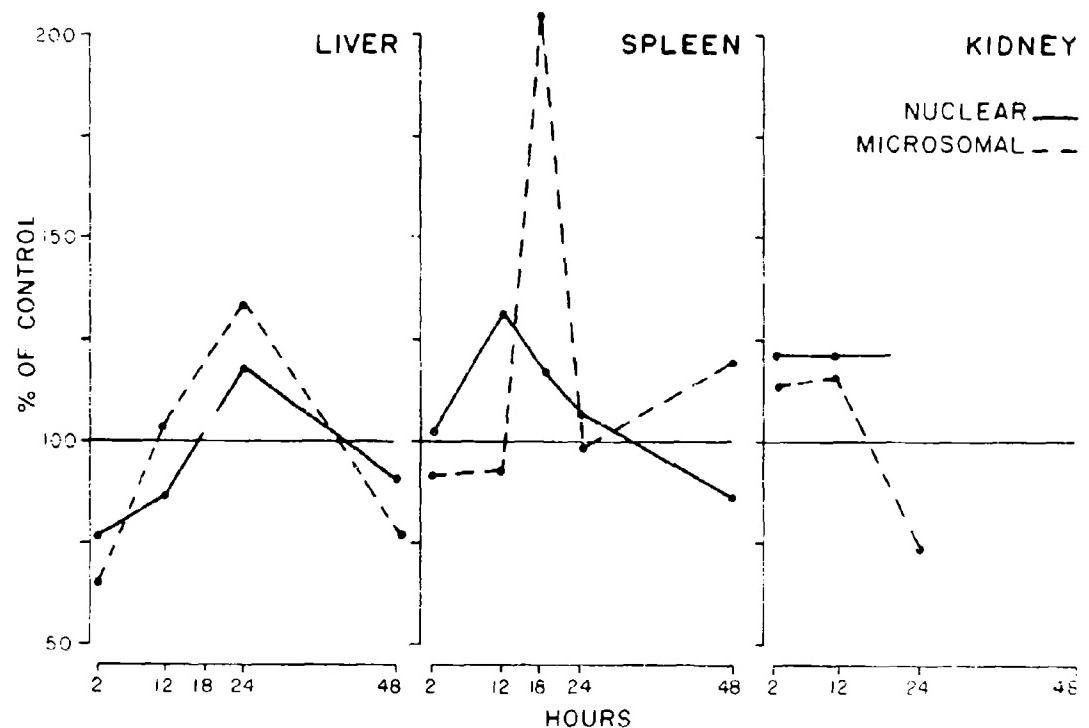


FIGURE 5. SPECIFIC ACTIVITIES OF RNA EXTRACTED FROM SUBCELLULAR FRACTIONS OF MOUSE TISSUES FOLLOWING PNEUMOCOCCAL INFECTION.

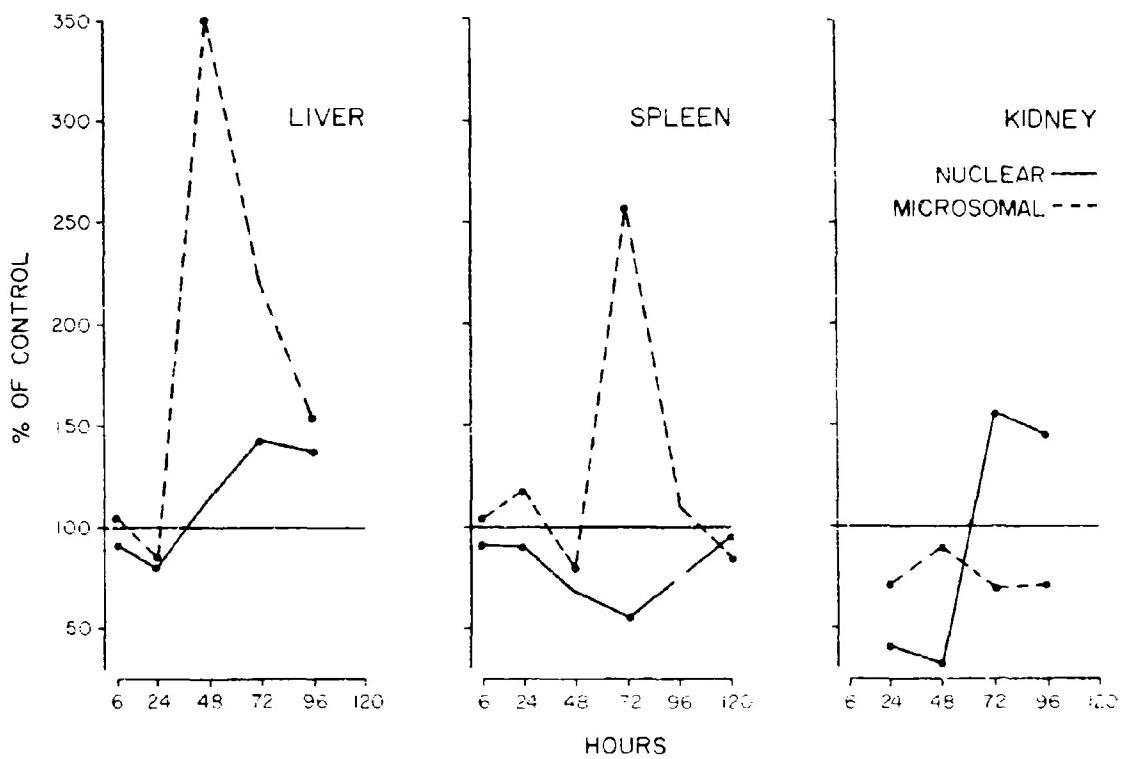


FIGURE 6. SPECIFIC ACTIVITIES OF RNA EXTRACTED FROM SUBCELLULAR FRACTIONS OF MOUSE TISSUES FOLLOWING VEE INFECTION.

The following observations may be of some significance:

(1) Although some correlation in direction and magnitude with previously observed alterations of protein synthesis was noted,^{1/} it was not total. This suggests that changes in protein synthesis patterns during disease are attributable only in part to precedent changes in RNA metabolism.

(2) Different organs showed different directions of change in the utilization of orotic acid at a given stage of a given disease. For example, as shown in Figure 5, liver microsomes showed an increased isotope content 24 hr after the initiation of the pneumococcal infection, while kidney microsomes showed a decreased content at this stage.

(3) During the early stages of the virus infection, nuclear preparations from all 3 organs showed a reduced isotope content.

(4) No matter which the specific patterns turn out to be, RNA metabolic alterations appear to occur in whole animals during infectious disease. These alterations are of sufficient magnitude, at least during some stage of illness, so that disturbances of normal cellular function would certainly result.

These studies have suggested that purified nuclear preparations and microsomal fractions deserve more detailed study. The continuation of these investigations will include attempts to detect altered polysomal function using density gradient procedures.

SUMMARY

Alterations in the utilization of a specific RNA metabolic precursor have been detected in groups of mice experimentally infected with D. pneumoniae or VEE virus.

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DISCUSSION

DR. MUNRO: Dr. Kehoe, have you measured or attempted to measure the amount of RNA per nucleus or have you considered the study of total RNA in the nucleus during these phases. The actual amounts can go down quite instantly with interference to RNA synthesis. For example, with actinomycin it would be recorded that there is quite a dramatic fall in the total RNA content of the purified nuclei, and similarly, there is an even more significant effect on the rate of polymerization from precursors. This would give you confirmation of the nuclear behavior which is otherwise subject to problems of size and things of this kind.

DR. KEHOE: These points are, of course, very good. We have not measured total RNA in the nuclei. We are following up our current studies and we will give serious consideration to incorporating that into any further work that we do.

DR. GRAY: What was the level of radioactivity in these samples? The reason I ask this question is that these fractions probably had virus in them. Since this is an RNA virus, how much of the activity could have been the result of viral contamination? If there was much, it could be very important.

DR. KEHOE: This is one of the big problems Dr. Levy was referring to this morning in whole animal studies, i.e., segregating the agent from the host, particularly with reference to one chemical moiety like this one we are using as a precursor. I can't really say what belongs to virus and what belongs to host at this point with the exception of nuclear activities. This is one thing that is restricted to the host since this virus does not localize within the nucleus. In terms of the levels - do you mean in terms of how many counts we get?

DR. GRAY: Yes.

DR. KEHOE: This seemed to be organ-dependent. The kidney would be thousands per OD, and the liver hundreds or low thousands per OD. But there was no possibility, using this criterion, to correlate it with known previous studies that have been done on the distribution of this virus in tissues by culture. Dr. John Tasker, you may recall, had done some of this in 1959 or 1960. He found the peak at about 24 hr in terms of his capacity to culture the virus. And the levels were maintained at quite high levels for some time. So this does introduce a lot of difficulties in terms of interpreting my observations relative to viral concentrations. I really couldn't go any further than that.

DR. LEVINE: What steps have you taken to insure that your nuclear preparation is clean? Second, what assurance do you have that you are not losing labeled RNA leakage from nuclear preparations? Third, what assurance do you have that the different preparations from different tissues are really comparable? That is, in one case, perhaps you have more contamination of nuclei with cytoplasm; more from kidney, for instance, than from liver.

DR. KEHOE: Well, I would just say that we developed a nuclear technique on the basis of some other observations, particularly the ones that had been developed by Dr. Penman. We did not examine them with an electron microscope, we did not look at them.

DR. LEVINE: Have you done any sucrose density gradients to see if there is any ribosomal RNA in nuclear fractions?

DR. KEHOE: No, we have not.

DR. LEVINE: I think this would be very important.

DR. KEHOE: The only thing I can say is that the work of the investigators who developed these methods show that in their system, which is principally liver nuclei, permitted recovery of purified preparations at the bottom of their discontinuous gradient. Visually we do get a lot of material sedimented, so it certainly does remove a lot, and the color is clear. We have not examined it except to take a look at it on the diagnostic microscope.

DR. LEVINE: You don't use any phase microscopy?

DR. KEHOE: We did look at it with phase microscopy in the liver, not in the kidney or spleen, and we could see no cytoplasmic tabs. But I think it's been shown that you can't really be sure you've gotten rid of all the tabs because the membranes go continuously into the cytoplasm. Principally we're depending upon the work of others. About your other two questions, it is certainly possible to get leakage from the nuclei. We have used fresh tissues all the time. We do no work with frozen tissues. Every batch of mice is a fresh batch. Tissues are processed immediately after excision and the differential centrifugation is carried out in the cold.

DR. LEVINE: I think leakage would arise mainly at the point of separating nuclei.

DR. KEHOE: Well, that certainly is a possibility.

DR. LEVY: Insofar as the question of how much of the radioactivity is accounted for by whole virus, I think it probably would not be a really significant amount -- with virus you really have to work at it to get a large number of counts. You could check, I suppose, by an immune precipitation by making an antiserum to your purified virus and try to precipitate out radioactivity but I don't think that's too much of a problem. Have you looked to determine the nature of the molecular species of RNA that is altered? Is it a ribosome or is it an 18S or a 28S, or is it possibly a messenger RNA alteration?

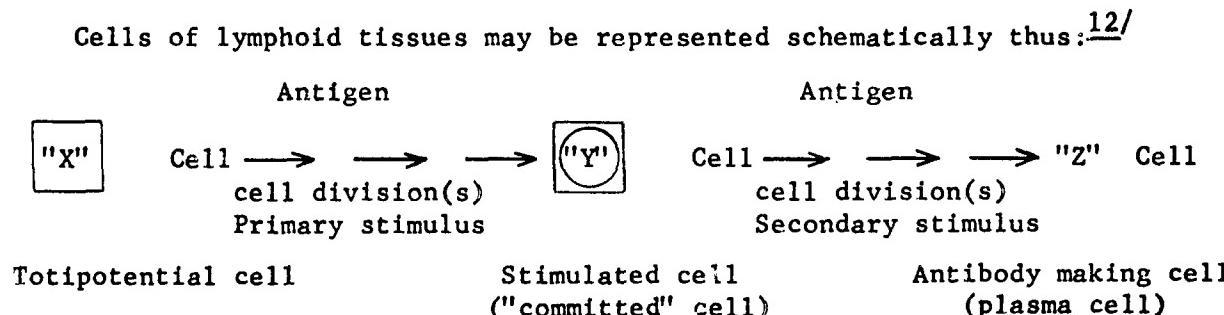
DR. KEHOE: This of course is a very good question. We have not progressed to that point. We have used Dr. Munro's agarose electrophoresis to some extent and we have used some sucrose gradients, but I have not gotten any results to date that would answer these specific and important points.

NUCLEIC ACID METABOLISM IN THE HUMAN LYMPHOCYTE

Hamish N. Munro, Ph.D.*

The small lymphocyte^{1/} is an immunocompetent cell capable of synthesizing in vitro RNA,^{2/} protein,^{3/} and DNA^{4/} and of dividing.^{5/} When stimulated in vitro with nonspecific "mitogens" (phytohemagglutinin (PHA), pokeweed extract, and staphylococcal exotoxin) the majority of lymphocytes in culture perform all these functions in a greatly accelerated fashion, transforming into enlarged, pyroninophilic (RNA-rich) cells.^{6/} Through proliferation and differentiation in the body, small lymphocytes probably participate in all aspects of immunity, including protection against infectious disease.^{7/} In the body it appears to be established that the small lymphocyte becomes the enlarged pyroninophilic cell (immunoblast) seen at sites of the homograft reaction^{8/} or with antibody formation.^{9/} The evolution from immunoblast to antibody-producing plasma cell in vivo, although accepted by most, has not been proven. Once established, this chain of events will place the small lymphocyte as the key cell behind most immune phenomena, including resistance to infection. Circulating small lymphocytes are a fairly representative sample of the total body lymphocytes, and are a convenient "pure" cell type which can be removed and studied in vitro. We have used thoracic duct lymphocytes, uncontaminated with other white cell types, in studies whose objective is to clarify the biochemical mechanism of transformation, and to link this mechanism with that of immune resistance. Ultimately, we wish to see whether nutrition of the host (the donor of the lymphocytes for in vitro studies) can modify the mechanism of transformation and therewith of resistance to infection. It would be particularly useful if some early in vitro event could be used as a test for the immunological responsiveness of the lymphocyte.

The theoretical model upon which we base this research is as follows: if the PHA effect is nonimmunologic as is favored by recent evidence^{10,11/} and the specific antigen effect is immunologic,^{6/} then it is reasonable to assume that the biochemical events associated with the lymphocyte response in each case are different as well, and can be affected differently by manipulation of the cellular environment (e.g., host nutrition).



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= Transformable by PHA



= Transformed only by "specific" antigens

If PHA stimulated most lymphocytes to transform, regardless of prior antigenic exposure, it should transform both X and Y cells, whereas each specific antigen can presumably transform only that subset of Y cells exposed to it in the past. Collectively, however, the Y cells probably make up most of the recirculating small lymphocytes of the body; in the rat, for example, the figure has been estimated as 90% for long-lived ("committed") small lymphocytes.^{13/} A widely held view of antibody induction is that the cellular mechanism for synthesis of antibody is "instructed" by antigen in a way that alters either transcription or translation to produce a particular protein (antibody), and that the cell retains this instruction throughout its lifetime.^{14/} Such a cell would be the "Y" cell in the scheme shown. Although morphologically indistinguishable from its "X" cell parent, the "Y" cell already has acquired the necessary information to produce specific antibody; it is presumably these "Y" lymphocytes, and only they, which transform in vitro in the presence of the antigen which originally "instructed" them. "X" cells do not transform in the presence of that "specific" antigen, but do transform if exposed to PHA, pokeweed extract, or staphylococcal exotoxin (nonspecific mitogens). If the assumption is correct that the difference in response of the X and the Y cells has a biochemical basis, then it should be possible to detect, very soon after exposure to the mitogens, biochemical differences between the responses of the X and Y cells to mitogens. Cooper and Rubin^{15/} studied labeling patterns of newly synthesized RNA in lymphocytes cultured with streptolysin O for 7 days and lymphocytes from the same patient in culture with PHA for 60 hr. From differences in sucrose density gradient labeling profiles, it was concluded that most of the rapidly synthesized RNA in the PHA-stimulated lymphocytes was nonribosomal (many species of messenger RNA?) whereas streptolysin O stimulated the synthesis primarily of ribosomal RNA. After exposure of lymphocytes to mitogens, very early changes in RNA metabolism must occur which prepare the cells for subsequent morphologically detectable changes. Although differences in RNA metabolism of cells may be measurable at all intervals after their exposure to PHA and specific mitogens respectively, the greater differences should be measurable soon after exposure, while the cells are in the process of differentiating. It has been reported that acetylation of histones^{17/} and phosphorylation of nuclear proteins^{16/} were both greatly accelerated within 15 min after exposure of lymphocytes to PHA. Kleinsmith et al.^{16/} postulate that negatively charged phosphate groups of the phosphoprotein might interact with the positively charged histones, thereby displacing the inhibitory

histones from the DNA-histone complex, thus allowing the DNA to become active as a template for RNA synthesis. Transcription of this "unmasked" DNA would permit the synthesis of the many new species of RNA required as the lymphocyte "transforms" or differentiates under the influence of PHA.

The present observations made use of the availability of a large supply of human lymphocytes from subjects who were being depleted of lymphocytes prior to kidney transplantation. These cells were studied during incubation with and without PHA. The first series of studies was intended to test (1) serially with treatment (immunosuppressive drugs and transplantation) the functional status of thoracic duct lymphocytes (TDL); and (2) the competence of these TDL to respond to PHA in tissue culture, using RNA content of the cell as a measure of response. In all patients the mean RNA/DNA ratio of "fresh" (unincubated) lymphocytes increased transiently as azathioprine (Imuran^R, Burroughs-Wellcome) or prednisone were administered. In a particular case, representative of 3 of 4 patients so studied, the RNA/DNA ratio of the TDL rose from 0.26 to 0.37 shortly after prednisone treatment was started. By a mechanism as yet not understood, adrenal glucocorticoids cause rapid lymphocytolysis with resultant involution of all lymphoid tissues and lymphopenia.¹⁷⁻¹⁹ That small lymphocytes are more susceptible to destruction than large ones²⁰ probably explains the promptly rising RNA/DNA ratio of the remaining (larger) lymphocytes. As the immunosuppressive drugs are continued, the ratio falls and finally stabilizes either above or below the pretreatment level. Corticosteroids are known to inhibit synthesis of RNA and DNA in lymphoid cells²¹ and in human lymphocytes;²² thus, the leukopenia and immunosuppression are maintained as prednisone is continued. With prolonged anathioprine and prednisone therapy after transplant, the ratio fell below the pretreatment level. This final decrease in the ratio probably indicated that in this patient the grafted kidney was less likely to be rejected in the near future than would be true if the ratio remained higher than the pretreatment one. The immunological potential of the TDL was examined by studying their response in culture to PHA in comparison to the response of control cells incubated in the absence of the mitogen. An experiment with cells obtained from patient W.S. before immunosuppressive treatment shows that the DNA content of the cultures fell with time, both with and without PHA, and signifies the cell death and dissolution which occurs in lymphocyte cultures. Despite falling cell numbers, there is net synthesis of RNA, but this only occurs when the cells are obtained before anathioprine treatment and cultured in the presence of PHA. The experiment also illustrates the results of a similar experiment with cells obtained from the same patient during the same therapy. These cells, exposed to the drug *in vivo*, but not *in vitro*, produced minimal synthesis of RNA in culture when stimulated with PHA. The effect of azathioprine treatment *in vivo* on the TDL to limit the action of PHA in stimulating net RNA synthesis was a reproducible finding observed in the 3 patients in whom it was tested. While there is clearly no net synthesis of RNA in the absence of the mitogen, the mean RNA/DNA ratio of the treated cells does not increase with PHA, although the rate of increase is slower than that in control lymphocytes. Despite a higher initial value, the ratio is at

a lower level than control cells after 60 hr incubation. The RNA/DNA ratio of cultured, untreated cells is remarkably stable, around 0.25, over a 60-hr period in the absence of PHA, while in cells exposed in vivo to azathioprine there is a steady decline in the ratio throughout the culture period. Thus, cultured TDL that had been exposed in vivo to the drug did not respond well to PHA, indicating that in vivo immunosuppression is correlated with decreased in vitro transformation of lymphocytes. Hersh and Oppenheim²³ reported that the in vitro response of the blood lymphocytes of patients receiving intermittent therapy with 6-mercaptopurine was reduced from 71% before treatment to 1.5% during treatment. Percentage transformation was used as the measurement. Our results confirm their observations by a biochemical technique with many advantages over morphological percentage transformations.

The second series of studies with TDL concentrated on the type of RNA synthesized in vitro in response to stimulation. The resting small lymphocyte contains RNA species which, like other mammalian cells tested, are separable into 4-6S, 18S and 28S fractions on sucrose density gradient fractionation.²⁴ We have found that the PHA-stimulated lymphocyte rapidly synthesizes new RNA species detectable by labeling the lymphocytes in culture with H^3 -uridine. Different species of RNA predominate at different time intervals after exposure to the transforming agent. The first workers in this field, Rubin and Cooper,²⁵ reported that mainly nonribosomal RNA was synthesized rapidly within the first 1-6 hr after PHA; ribosomal RNA was detectable after 6 hr and was synthesized in increasing amounts for the duration (usually 72 hr) of PHA exposure, but even at 40-60 hr after PHA, nonribosomal RNA predominated. In contrast these same workers found that lymphocytes exposed to a specific antigen, streptolysin O, produced mainly ribosomal RNA after 7 days incubation.¹⁵ The "nonribosomal" RNA found 1-6 hr after PHA could well have been a degradation product of a higher molecular weight (MW) RNA, however, since it is difficult to extract undegraded newly synthesized RNA from either resting or PHA-incubated lymphocytes. PHA may induce, among other things, a ribonuclease which degrades the high MW RNA during extraction. Whatever the reason, the lability of the newly synthesized RNA has resulted in a number of conflicting reports. We have been able to circumvent the problems of RNA degradation by employing a technique for rapid extraction of RNA used by Dr. Jacob of this laboratory.²⁴ The lack of RNA degradation in our preparations is indicated by the fact that label appears in high MW RNA fractions (40-50S) but not lower MW fractions in the same gradient. Thus, unlike previous workers, we have been able to show that PHA stimulates, within 1-2 hr, the synthesis of high MW RNA, some of which is ribosomal-RNA precursor. Using TDL from patients about to receive renal transplants, we have studied RNA metabolism during the first 2 hr after exposure to PHA. Washed TDL are cultured in medium 199 at 37 C with 3% autologous serum, with and without PHA. The suspensions are then pulse-labeled for 30 min with H^3 -uridine. RNA is extracted either immediately with hot phenol or after a 1 hr chase with actinomycin D and cold uridine which stop RNA synthesis and allow the newly synthesized, labeled RNA in the cells to

be processed. Results of RNA extraction from whole cells following 60-80 min incubation showed that PHA stimulates the synthesis of 4-6S and of 45-50S RNA. The kinetics of labeling were then determined by pulse labeling pairs of tubes for 2, 5, 10, 20 and 30 min, the timing adjusted so that the duration of exposure to PHA was equal (80 min) in all tubes. H^3 -uridine was incorporated rapidly into 4-6S RNA with a peak detectable after the 2-min pulse and continued to rise with increasing labeling time. Radioactivity peaks were 50-100% higher in the PHA tubes than in control tubes. Label was incorporated more slowly into the high MW RNA (45-50S); radioactivity, first detectable at 10 min, continued to increase in this fraction. The incorporation into this high MW species was then examined in more detail using isolated lymphocyte nuclei. After the incubation procedures, lymphocyte nuclei were isolated by treatment with 0.01 M citric acid⁴ and nuclear RNA was extracted. This served the dual purpose of eliminating the cytoplasmic ribonuclease and concentrating the high MW newly synthesized RNA. PHA -incubated lymphocytes synthesize from 50-100% more high MW nuclear RNA (45-50S) than do lymphocytes from the same patient incubated without PHA. Much of this nuclear RNA appears to be ribosomal RNA precursor, since it is chased to a peak at 28-32S. Thus, our results disagree with those of Rubin and Cooper, probably because of our minimal RNA degradation during the extraction procedure. Some of the newly synthesized high MW RNA is probably not ribosomal RNA precursor and may be nuclear heterodisperse, non-nucleolar 45S, or other RNA. Studies are now in progress to determine the nature of these high MW RNA species.

SUMMARY

The mitogen-stimulated small lymphocyte is a model with which cellular differentiation can be studied *in vitro*. The immune responses of the small lymphocyte *in vivo* are believed to be reflected by the degree to which this cell can be transformed *in vitro* into an enlarged blastoid form which actively synthesizes RNA, protein, and DNA. Reported herein are biochemical changes in the stimulated lymphocyte which are shown to reflect in part the immune history and status of the host (donor of the lymphocytes). It is highly probable that the host's immune status (e.g., resistance to infection) and in turn the biochemical events of very early transformation of his lymphocytes may be altered by the nutritional status of the host. The present investigations are exploring these possibilities with the ultimate objectives of (1) learning more about the biochemical events associated with the responses of immune cells and how to demonstrate them readily and (2) developing practical tests which will permit evaluation of host factors (e.g., state of nutrition of host) as they modify the immune response.

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DISCUSSION

DR. KEHOE: Have you found endogenous nucleases to be particularly troublesome in these lymphoid tissues? If so, how might one circumvent these problems?

DR. MUNRO: We haven't actually looked for ribonuclease activity. First, we use the same isolation procedure which you are using, with slight modifications. Second, the ribonuclease is probably less serious in studies of purified nuclei because we got rid of cytoplasm. Third, we obtain very high molecular weight material. This suggests that we have been successful in avoiding ribonuclease activity, since if this activity were high, the large molecules would be reduced to a small size. Instead, they have survived the treatment; this in itself is evidence for the lack of troublesome endogenous nucleases.

DR. ZAMECNIK: Do you have any information about the base ratio or possible hybridization of this RNA?

DR. MUNRO: No. We hope to do several additional things in this area. Hybridization is certainly one thing included here, also, amino acid incorporation-stimulation in an Escherichia coli system, although that is perhaps less in favor than it was a year ago.

DR. KLAINER: Do small lymphocytes in normal subjects behave the same as the small lymphocyte in a patient about to have a renal transplant? It is known that a patient with uremia has an abnormal immune response; it may not be valid to equate the response of uremic lymphocytes with those from a normal individual.

DR. MUNRO: I can't answer that question from our own experience. The type of stimulation obtained in tissue culture with and without phytohemagglutinin treatment appears to be similar to that reported by other groups. In other words, the effect on cell divisions and on cell numbers was similar.

DR. LEVY: How well could you differentiate between a 4S and a 5S RNA? I thought I could see what looked like a constant shift about 1 or 2 tubes higher in radioactivity in your graphs. Was this the mysterious 5S rather than a 4S RNA, or was this my imagination?

DR. MUNRO: I would agree that it is extremely difficult to differentiate these components even if you are collecting drops from the tubes.

DR. LEVY: After seeing these figures, one gets some perspective.

DR. MUNRO: Yes, it is quite possible. I mentioned that one worker in Cambridge, England, has claimed in an unsubstantiated abstract that the fit of the nitrogen in the 4S region is not due to attachment of sRNA or transfer RNA. So it is possible that something is happening there. Of course, even if it is a 5S particle of RNA that is stimulated, we know nothing about its function, even though we know its structure.

DR. LEVY: It just might be worth knowing, if that's the case.

DR. MUNRO: We should really use what Dr. Kehoe referred to, namely, our own gel technique to look at these things.

DR. ZAMECNIK: In relationship to the small lymphocyte and the contribution of its nucleus, one should mention the interesting work of Henry Harris who studied the duck erythrocyte which has a nucleus but one that is pretty well "turned off." It normally doesn't do anything when studied, but if put in contact with the macrophage from rabbits in the presence of an irradiated virus, phagocytosis occurs. Then, both nuclei are within the same cell. Under such circumstances, the duck erythrocyte nucleus becomes

"turned on." In interpreting such a finding, it would appear that some message comes from the active DNA-producing nucleus. As a result, the duck red cell nucleus "comes apart," enough so that it's no longer repressed and can then begin to synthesize DNA.

NUCLEIC ACID SYNTHESIS AND MICROCONTAMINANTS IN TISSUE CULTURE

(Abstract Only Submitted)

Elliot M. Levine, Ph.D., and Harry Eagle, M.D.*

Many cell cultures in our laboratory appear to develop profound changes in the pattern of nucleic acid synthesis during their serial propagation.^{1/} The specific alterations are:

(1) Appearance of a New Species of RNA.--"Altered cultures synthesize a rapidly labeled cytoplasmic RNA which has a sedimentation constant of 14-20S and a guanine plus cytosine content of 33%. Also, in contrast to RNA synthesis in normal diploid cells, synthesis of the new species in "altered" diploid cells is not markedly decreased in confluent cultures.

(2) Absence of C¹⁴-Uridine Incorporation into Ribosomal Precursor RNA.--Normal nuclear incorporation of C¹⁴-uridine into ribosomal precursor RNA (35-4S3) is depressed to the degree that it can no longer be demonstrated either by autoradiography or sucrose density centrifugation. Although incorporation into ribosomal RNA does occur after prolonged exposure (24 hr) to C¹⁴-uridine, the estimated rate of ribosomal synthesis is < 1/3 the normal value.

(3) Appearance of a New Species of DNA.--Paralleling the synthesis of rapidly labeled 14-20S RNA in altered cells, a new species of DNA appears, also synthesized in the cytoplasm and separable from normal DNA by sucrose density centrifugation. Autoradiographs of altered cultures pulsed with tritiated thymidine reveal few, if any, cells incorporating isotope into normal nuclear DNA.

Despite these profound changes in nucleic acid synthesis, the gross appearance, generation time and life expectancy of these altered cultures are not demonstrably affected. The observation that supernatant culture fluid from altered cells often effects a similar alteration of previously normal cells, implicates a noncytopathic microcontaminant as the cause of the foregoing changes.

Preliminary experiments suggest that inoculation with Mycoplasma can produce changes in cell cultures similar to those described above. Studies are continuing as to the nature of Mycoplasma-cell interaction in culture with regard to nucleic acid synthesis.

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DISCUSSION

DR. WOODWARD: Thank you Dr. Levine. I think this a beautiful example of an instance where the biochemist needs to know some microbiology. We have many examples in the other direction. This is a lovely one in this direction.

DR. LEVINE: Unfortunately we were content to send these samples out to industrial labs for analysis. It is quite evident to people in the Mycoplasma field that such tests are just not sensitive enough to detect Mycoplasma all the time. Furthermore, we are still mystified as to why there is only positive correlation between mycoplasma and the abnormal RNA. That is, we have tested several cultures known to be contaminated with PPLO and yet these have a normal pattern of RNA synthesis. So---I rest my case.

DR. LEVY: Despite the little bit of tragedy in this, it also is a very interesting fact that these contaminated cultures are synthesizing cellular RNA at a much reduced rate in contrast to the noncontaminated cultures which seem to be growing normally.

DR. LEVINE: Well, this is a very important point. Actually there must be synthesis of ribosomal RNA in these contaminated cultures just as there must be synthesis of cellular DNA in these cultures and yet we detected no incorporation of precursor. This very point emphasizes the need for caution in interpreting all tracer work.

Three possibilities exist: First, utilization of the tracer by the PPLO. This seems unlikely, since investigators in the past have added culture fluid to a normal culture after a pulse and gotten incorporation. So we can probably eliminate this. Second, is some sort of repression of the mixing of exogenous tracer with the endogenous pools in the cell? Now we know there can be all kinds of compartmentalization in the cell and it would be interesting if Mycoplasma somehow affects the entrance of exogenous uridine into the endogenous uridine pool. Third, we could have a generalized disruption of the cell permeability. This seems rather unlikely because the cells continue to live and they continue to require essential amino acids and other nutrients which are provided through the medium.

Two of these possibilities may be combined in an interesting manner. If somehow Mycoplasma were affixed all over the cell surface it is entirely possible that exogenous, labeled uridine has to pass through so much Mycoplasma that it just never manages to get into the cell. And yet the cell,

being able to synthesize both pyrimidine and uridine, continues to grow and produce ribosomes.

DR. LEVY: Maybe that is the whole crux of the thing, that it can't use synthesized uridine but instead is required to make its own uridine. What about phosphorus for example as a tracer for RNA synthesis instead of uridine?

DR. LEVINE: Phosphorus is incorporated into the abnormal RNA without incorporation of significant traces of uridine as a ribosomal precursor. It is also possible to utilize C^{14} methylmethionine as a precursor for labeling nucleic acids. Since this is an amino acid and an essential one for tissue culture cells, it must get into the cell. We hope to study host cultures with both methylmethionine and uridine in a double isotope experiment to solve the problem.

DR. KEHOE: In terms of some of the inconsistencies here, one must keep in mind the difficulties in characterizing Mycoplasma, even among experts. This is pointed up in a symposium published by the New York Academy of Science, based on a meeting about a year ago. A number of criteria can be listed that will allow a microorganism to be characterized as a Mycoplasma. But this group is widely divergent, especially ones found in animals compared to ones found in sewage.

DR. LEVINE: Our recent studies of the Mycoplasma have been followed up by identification with specific antisera so we are fairly sure of what we are dealing with at this point. This is one advantage of collaborating with experts.

DR. KEHOE: What species have they been?

DR. LEVINE: Well, this is a very curious thing. About 5 or 6 years ago, the most common PPLO found in tissue culture was Mycoplasma orale but now 90% of all tissue cultures are contaminated with Mycoplasma hyorhinus, otherwise known as the GBL strain, a strain which is supposed to have originated in swine. People have hypothesized that the trypsin used in preparation of cultures was the source of this contamination, but these have been tested for PPLO and are consistently negative. You can invent the "fairy tale" that a batch 5 years ago was prepared from swine with GBL and that all the stocks became contaminated with GBL which now spreads throughout the labs by aerosol.

DR. WOOD: Can you see this Mycoplasma under the electron microscope in the infected cells?

DR. LEVINE: This is another point that mislead us. Certain Mycoplasmas stain very densely when seen under the electron microscope. Mycoplasma such as GBL bear a very close resemblance to cytoplasmic inclusions when they are present at the cell surface. In retrospect we can pick out

electron micrographs of these cultures and say that this must have been contaminated, but even so, the interior of the cell looks perfectly normal. There is no disruption of mitochondria, no abnormal amount of lysosomes in the cell, just what appears to be artifactual surface processes which in retrospect may have been GBL Mycoplasma. I frankly don't think that electron microscopy is a very sensitive tool for this when dealing with GBL.

THE POSSIBLE USE OF OLIGONUCLEOTIDE ANALOGUES AS VIRAL INHIBITORS

Paul C. Zamecnik, M.D.* , and Orrie Friedman, Ph.D.**

The induction in a host cell of an RNA-dependent RNA polymerase by a viral invader presents a special chemotherapeutic challenge. If an oligonucleotide can be found to inhibit this enzyme while sparing the synthetic action of the normal DNA-RNA polymerase of the cell, the reproduction of the virus may be brought to a halt, while the host is spared. There is, however, to our knowledge, little work on the effect of oligonucleotides as possible viral RNA inhibitors. Dinucleoside monophosphates containing 5-fluorouridine have been tested recently as potential anticancer agents, and a current review^{1/} pulls together scattered information on the biodynamic effects of oligonucleotides.

Biochemically speaking, viruses are of two types: DNA oncogenic viruses and RNA oncogenic viruses. In the case of the former, a small amount of viral DNA appears to be incorporated into the genome of perhaps one in a million of the host cells, thus producing a mutation which can be carried through successive progeny of that single cell without the necessity for further viral intervention. That situation does not, however, apply to the RNA oncogenic viruses. In the avian myeloblastosis and erythroblastosis viruses (both of which are RNA leukemia viruses), there are continually high concentrations of virus present in the plasma of the affected animal until the time of death. It is likely that sustained production of viral RNA is essential in the maintenance of an RNA viral leukemia. As shown in Figure 1, competition of viral RNA and host cell messenger RNA for monoribosomes may occur at the translation step. Thus viral RNA competes with cell messenger RNA for the monoribosome pool.

One of the important proteins synthesized under instructions from the viral RNA is an RNA replicase (RNA polymerase), an enzyme not found in the uninfected cell. This new type of enzyme, plus the viral RNA itself, would be specific chemotherapeutic targets.

Toward such a goal, we thought it desirable to synthesize oligonucleotides which had mixed components, i.e., ribo- and deoxyribonucleotides. One is in part thinking of hydrogen bonding competitors, and we have information from the work of Nirenberg et al.^{2/} and Khorana and co-workers^{3/} that in the case of oligonucleotides with natural 3'-5' phosphodiester linkages, at least a trinucleotide is needed to serve as a good hydrogen bonder with a polynucleotide at body temperature.

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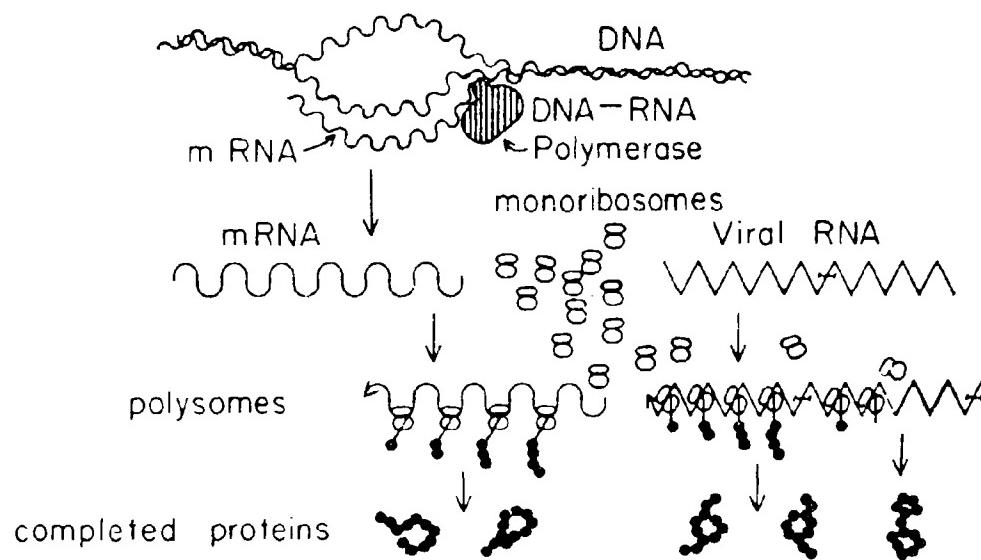


FIGURE I. COMPETITION OF mRNA AND VIRAL RNA FOR POLYRIBOSOMES IN PROTEIN SYNTHESIS.

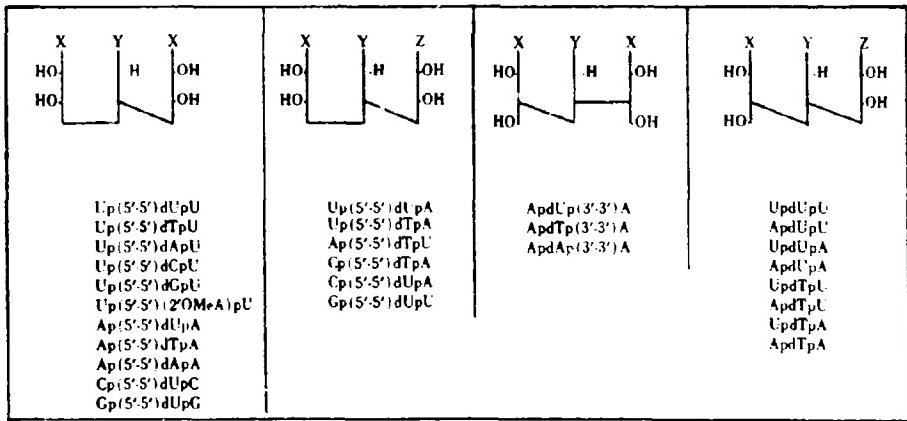
We therefore set out to synthesize unnatural dinucleotides, trinucleotides, and aimed toward eventual synthesis of longer oligonucleotides. After a great deal of effort, a number of mixed oligonucleotides were synthesized (Figure 2). Most of these contained U's and A's, because nucleosides containing these bases were the easiest to condense from suitably blocked monomer units. Synthetic oligonucleotides, both normal and abnormal, were thus prepared. The simplest deviation from a normal ribonucleotide is to preserve the usual 3'-5' internucleotide linkages, but to replace the central ribosome sugar moiety by deoxyribose. These compounds have been synthesized in the milligram amounts necessary for testing in in vitro systems which employ RNA replicase or the control DNA-directed RNA polymerase.

As shown in Figure 3, there are at least 5 points at which such oligonucleotide analogues could act, once having entered the cell. The cell entry question is an untested one at present, since in vitro cell-free systems are being used. They might act at the DNA-DNA replication level (point 1, Figure 3) or at the DNA-RNA transcription level (point 2) both of which are present in normal cells. This would not be a promising situation. We are using the Chamberlin and Berg^{4/} DNA-RNA polymerase, prepared from normal Escherichia coli, to test for inhibition at this point. This represents the control enzyme assay. The oligonucleotides could however hopefully compete at points 3 or 4, which are specific for the virus invader. These are the sites toward which the oligonucleotide synthesis has been directed. In the test RNA viral infection (i.e., QB phage infection of E. coli) one might hopefully have a competition of oligonucleotide analogue with the viral messenger RNA for attachment to viral RNA replicase (point 3). This would be a competition between oligonucleotide and viral RNA for the position as template in the replicase reaction.

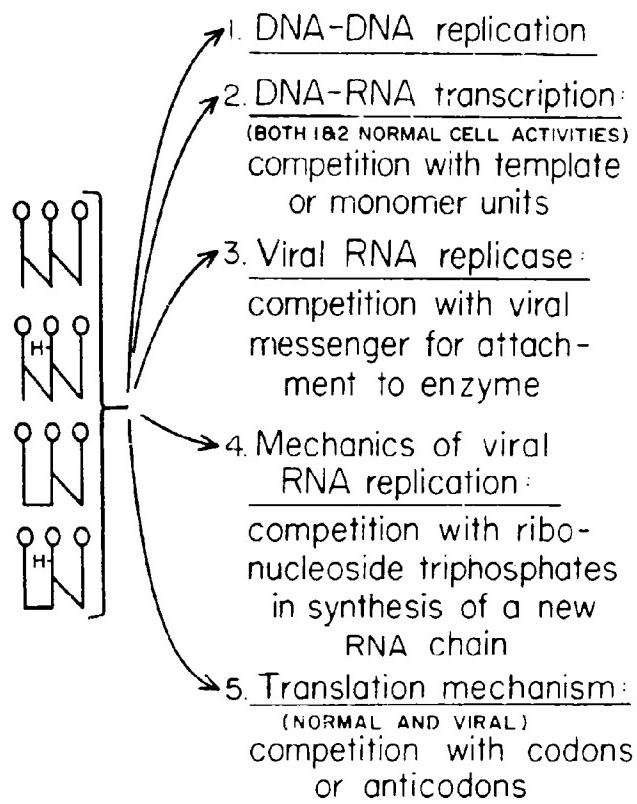
As mentioned in point 4, the oligonucleotide might also compete with ribonucleoside triphosphates in the actual construction of a new viral RNA chain on the viral RNA template. Selective action of oligonucleotide analogues at points 3 or 4 would be the ideal situation for chemotherapy.

To complete this analysis of possible sites of intracellular action of oligonucleotides, there might also be interference at the translation step, i.e., a competition of oligonucleotide with codon or anticodon (point 5, Figure 3). At this point, it would be unpredictable whether the virus or the host translation-mechanism would be more affected.

The enzymes we chose to test in this in vitro system were QB RNA replicase and the MS 2 replicase, initially as prepared according to Spiegelman but more lately as prepared according to our colleague on this project, Dr. J. T. August.^{5/} Important to the possible success of this project, Dr. August's preparation of QB replicase requires the addition of a primer, in order to synthesize RNA from a mixture of the 4 ribonucleoside triphosphates. It is a key requirement to have an enzyme preparation which needs added RNA for its activity, since it makes possible the search for a competitive effect of oligonucleotide analogues at point 3, Figure 3. If an



**FIGURE 2. MIXED OLIGONUCLEOTIDES
SYNTHESIZED.**



**FIGURE 3. POSSIBLE INTRACELL-
ULAR ACTIONS OF
OLIGONUCLEOTIDES.**

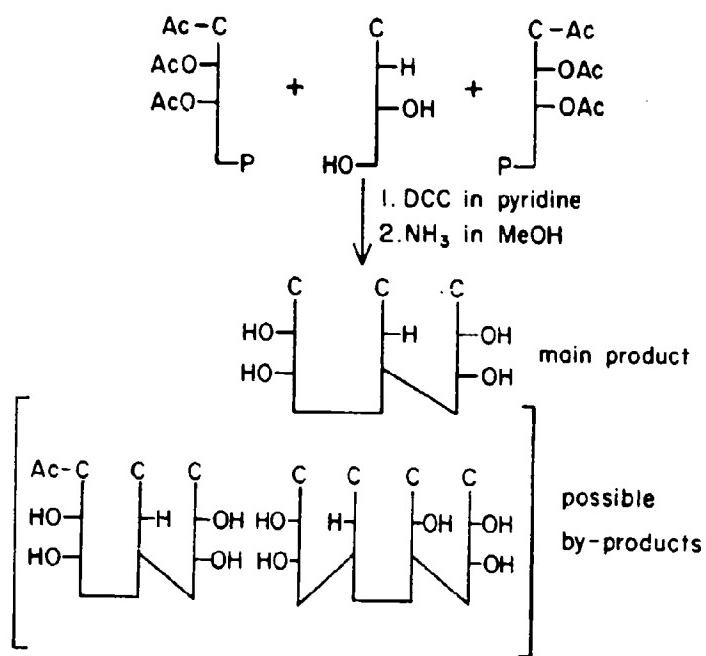


FIGURE 4. SYNTHESIS OF A TRI-NUCLEOTIDE.

oligonucleotide were found to inhibit this QB replicase (points 3 and 4) while sparing the action of the normal cell DNA replication and transcription enzymes (points 1 and 2) we would have a promising lead. We therefore prepared the normal E. coli transcription enzyme (point 2) as well as the QB replicase for this type of test.

The compounds previously shown (Figure 2) were synthesized with two things in mind. In the first place, they were technically the easiest trinucleotides to make; and second, one didn't have any information as to which nucleotides were present at the start of the viral RNA chain and which were last in the viral RNA chain. Recent data from Dr. August's laboratory^{5/} now indicate that there is pppGG on one end of the QB RNA. On the other end, there is some suggestion for the presence of -CCA. In other phage RNA's the presence of PG, ppG, or ppGG has also recently been found. It therefore appeared pertinent, in view of these recent data, to synthesize a trinucleotide which would hydrogen bond with the terminal GG - in other words, to synthesize CCC or CdCC or some variant containing C residues. In Figure 4 is shown the synthesis accomplished by Dr. Nicholas Starkovsky of Collaborative Research, Inc. First the free hydroxyl groups and the amino group on the C ring itself were acetylated, to produce acetylated cytidylic acid. Then a proportion of 2 molecules of triacetylcytidylic acid to 1 molecule of deoxycytidine were condensed with dicyclohexylcarbodiimide (DCC), and deacetylated with ammonia. The main product (2/3) consists of CdCC (Figure 4). The remaining 1/3 cannot be identified completely at the present time. Both the known main product (which may still contain minor unidentified compounds associated with it) and the unidentified mixture of several compounds inhibit the QB RNA replicase system at a concentration of 10^{-4} - 10^{-5} molar, while not inhibiting the control DNA-RNA polymerase at the same concentrations.

SUMMARY

The experimental story rests here at the present time. The groundwork for testing oligonucleotides has been laid, and the critical enzymes have been prepared in a way that they can now be frozen and remain active over long periods of time. It remains for oligonucleotide analogues to be identified more precisely and then to be tested in a rigorous way.

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DISCUSSION OF SECTION II

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We have heard data concerning specific instances of alterations in nucleic acid metabolism: some induced by infection, some induced by chemical agents, and some perhaps related to age or condition of cells. The papers have been discussed individually and I don't really think that I can or should discuss them individually any further. But perhaps it would be useful to bring up some "old-hat" material as a note of caution and then to discuss some other aspects of nucleic acid alteration. The relationship to these specific instances already discussed this morning will be apparent as we get along.

First, I will mention the relationship between the metabolic state of the cell and the types of alterations seen. In an early report now more than 10 years old, Wilbur Ackerman stated that infection of HeLa cell cultures by polio virus led to an increase in cellular RNA synthesis. Shortly thereafter just the opposite results were reported by Salzman and co-workers. At about the same time Sam Baron and I sometimes were finding an increase and sometimes a decrease in cellular RNA synthesis. After much heated discussion it developed that Ackerman was using confluent resting cells, characterized by a low endogenous rate of RNA synthesis, Salzman was using rapidly growing suspension cell cultures and Baron and I were not being so careful. Sometimes we would use fast-growing and sometimes slow-growing cells. But a dramatically rapid inhibition of cell RNA synthesis induced by Mengo virus was shown in suspension cultures of L cells. If one took suspension cultures of L cells, infected them with Mengo virus, and measured RNA synthesis by the incorporation of uridine into RNA, one noticed very rapidly a marked inhibition of RNA synthesis. Within 30 or 40 min after infection there was a marked decrease in cellular RNA synthesis. Concurrent with a decline in cell synthesis was a decrease in the nuclear DNA-dependent RNA polymerase as well as a decline in the polysomes devoted to cell protein synthesis. More recent data suggest that analogous conditions may apply to at least one DNA-containing virus, polyoma virus. In general it has been found that infection with polyoma virus increased cellular DNA synthesis. These studies by Dulbecco et al. and Roger Weil were done with resting, confluent cells. Rose Schlein and her group working with growing cells found inhibition of cellular DNA synthesis. Certainly in the studies with whole animal infection, a finding of an increase or decrease in cell RNA or DNA synthesis may merely imply that one probably is, in some cases, dealing with growing tissues; while in some cases one is dealing with tissues which have a very low endogenous rate of RNA synthesis.

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It might also be appropriate to present here some data on the effect of the states of cell metabolism and cell age on nucleic acid synthesis rates without reference to infection. Our experiments were done primarily to test the effects of interferon on cell RNA metabolism and used chick embryo cells to measure RNA and protein synthesis. RNA or protein precursor was added for a short period of time and the specific activity of the resultant RNA and protein was measured. Within 45 hr after initiating the cultures, the early specific activity of the RNA of 897 fell to 300, representing a marked decrease in the rate of RNA synthesis. This is primarily attributable to a decrease in cellular ribosomal RNA. On the other hand, the rate of protein synthesis did not decline. So that such cells were making protein despite the drop in RNA synthesis. This can also be expressed as a ratio of the rate of protein synthesis to the rate of RNA synthesis. Despite a falling RNA turnover in an aging culture, you get more protein synthesis per unit of RNA.

Close to my own interest is another type of nucleic acid alteration induced by many viruses upon infection of many different kinds of cells. For convenience I will call this a derepression of a host cell "cistron," but I am not sure that this is really a justified term. However, if one infects cells, such as chick embryo tissue culture, with any of a number of viruses, shortly thereafter one finds in the tissue culture fluid a new protein called interferon. There is an increase with time in the concentration of this protein. The production of interferon is blocked by actinomycin and also by the addition of inhibitors of protein synthesis. This is interpreted by most people (although it is not rigorously established) to mean that a suitable virus infection leads to the production of new messenger-RNA under the control of a host-cell gene, and consequent synthesis of the protein interferon.

Assuming that hypothesis, the following kind of experiment provides some idea of when the messenger-RNA for interferon is made. Cells are first infected with virus and at various times thereafter actinomycin is added to block further cell RNA synthesis, although synthesis of virus continued. The cell culture is allowed to incubate; the final interferon concentration is measured. One reason that interferon found at the end of incubation was produced from the messenger-RNA that had already been transcribed at the time the actinomycin was added. Actinomycin is added to block the synthesis of cellular RNA but virus synthesis is not blocked. In such a system, if actinomycin is added 1½ hr after infection, no interferon appears. If one adds actinomycin 2 hr after infection some interferon appears, and if it is added at 2½ hr a full yield of interferon appears. Therefore, one can state that the interferon that finally appeared was translated from the messenger-RNA present at the time that the actinomycin was added. And so the messenger-RNA for interferon production by a cell appears shortly after 1½ hr following initiation of infection. Thus, virus infection alters cell metabolism in such a way as to lead to the formation of a new species of cellular RNA and protein

that had not been previously in existence. As you know, when this interferon is added to other cells, the other cell will become resistant to subsequent virus infection.

The molecular mechanism of this resistance to virus infection may involve still another "derepression of host cell genome function." At any rate, cellular RNA and protein synthesis is needed after interferon application in order for the treated cell to develop viral resistance. Associated with this interferon-mediated resistance is the appearance of a new type of ribosome and a ribosome subunit that binds and translates cellular messenger-RNA normally, but viral RNA very poorly. We measured the endogenous translation activity of ribosomes prepared from control cells and cells that had been treated with interferon using an amino acid incorporation system, and found them to be equal. Further, priming by polyuridylic acid is the same for both types of ribosomes. Thus, the translation of normal and synthetic messenger-RNA was the same for both types of ribosomes. This was not true for viral RNA. When the endogenous rate of translation by control ribosomes and interferon type ribosomes was followed by priming by Mengo virus RNA, the control ribosomes showed normal translation; the interferon type ribosomes were found to be virtually zero after priming by Mengo RNA. In connection with this, there appears to be a compositional difference between the control and interferon type ribosomes in the ratio of protein to RNA. We don't know yet whether or not there are differences in the composition of this protein or the RNA. So here we have a host defense mechanism that is triggered by virus infection and is associated with an altered cell RNA synthesis. Phytohemagglutinin, which was discussed by Dr. Munro, also is capable of inducing interferon synthesis by lymphocytes and most likely by a mechanism analogous to that carried out by a virus. Double stranded RNA also does this. Upon treatment of cells with certain forms of double stranded RNA, interferon appears.

It would seem that a number of conditions can alter cell RNA synthesis to lead to the derepression of a repressed host cell genome function. Interferon itself appears to be analogous to certain protein hormones, in that they also seem to act to stimulate the formation of a messenger-RNA and a specific protein. It is rather hard to summarize such a diverse collection of responses and I shall not try to do so but it is obvious that these responses deserve considerable additional study.

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SECTION III

IMMUNOLOGICAL ASPECTS

MODERATOR: Dr. W. Barry Wood

DISCUSSANT: Dr. James G. Hirsch

DETECTION OF EARLY ANTIBODY

Captain Robert E. Krisch, MC*

The primary goal during the studies reported here has been to learn more about the primary immune response at the cellular level by identifying and counting individual antibody-producing cells and by studying biochemical changes in these cells at various times following antigenic stimulation. A secondary goal has been to evaluate the usefulness of the techniques developed and the information obtained for direct use in the early diagnosis of infection.

The two major problems encountered in this kind of study are: first, how to identify individual cells in lymphoid tissue or blood which produce antibody to a specific antigen, and second, how to study biochemical changes in single cells once they are identified.

The test system was determined primarily by the problem of identification of antibody-producing cells. For this purpose we decided to use the antibody plaque technique, developed by Jerne et al in 1963.^{1/} In all of our experiments, male, Fort Detrick strain, mice, weighing 25-35 gm, were immunized intravenously (IV) with sheep red blood cells (RBC). Mice were sacrificed at various times following this stimulus, their spleens were removed and cell suspensions in tissue culture medium were prepared by gentle homogenization and filtration through a fine screen. Spleen cells producing antibody to sheep RBC were then identified by means of the antibody plaque technique. Viable spleen cells are mixed with sheep RBC and molten soft agar in tissue culture medium. The mixture is incubated for 1 hr at 37 C, while the agar hardens. Complement is added in the form of guinea pig serum and incubation is continued for another half hour. Circles of hemolysis (0.1 to 0.5 mm in diameter) appear concentrically around those mouse spleen cells producing specific antibody to the sheep RBC. We have modified the technique for use with microscope slides rather than Petri dishes by spreading a thin layer of the molten agar cell suspension on a slide and incubating it in a 100% humidity chamber. After development of the plaques the slides are dried in air and fixed in methanol before subsequent staining, autoradiography, or other treatment. Figure 1 shows a Wright-Giemsa stained plaque with a well defined central cell.

The antibody plaque technique has significant advantages for studying the immune response at the cellular level: (1) It is extremely sensitive. A single antibody producing cell, which is the smallest discrete unit of antibody production, can be detected in a population of 10^5 or 10^6 spleen cells. The plaque technique detects antibody which is produced while the test is being run and did not exist when the spleen was removed. (2) It

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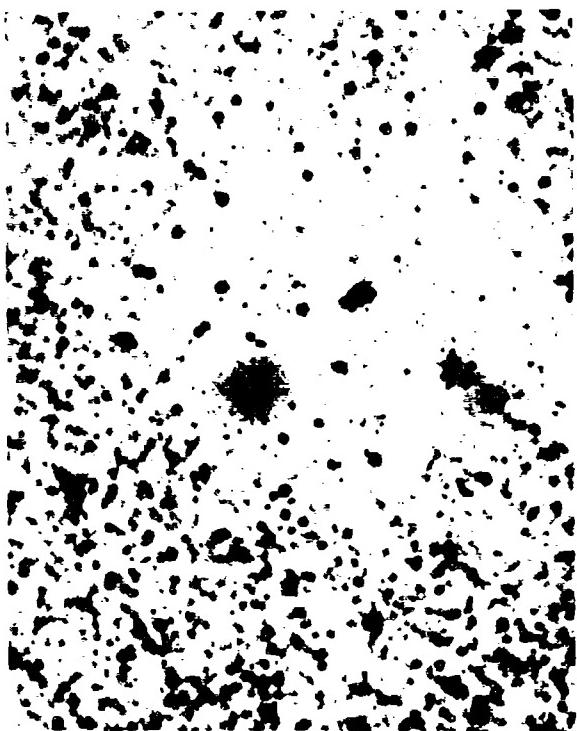


FIGURE I. ANTIBODY PLAQUE PREPARATION FROM ISOLATED MOUSE SPLEEN CELLS WITH WELL-DEFINED CENTRAL CELL (WRIGHT-GIEMSA, 72.5 X).

is highly specific for the antigen under study. There is no significant increase, for example, in the number of spleen cells producing plaques to chicken RBC in the spleen of a mouse immunized with sheep RBC. (3) Finally, the technique is relatively rapid and simple to carry out and gives readily producible results.

Our studies of the primary immune response have nearly all involved the use of antibody plaque preparations on microscope slides, made as described from spleen cell suspensions of mice immunized IV with sheep RBC.

The work falls into 3 categories: First, the pattern of change has been studied in the numbers of specific antibody-producing cells in the spleen following primary antigenic stimulus. Also, similar studies have been carried out on the appearance of immunocompetent cells in blood under the same conditions; second, DNA synthesis by immunocompetent cells in the spleen has been studied by combining the antibody plaque technique with autoradiography; and third, RNA content of immunocompetent cells has been studied by combining the plaque technique with the use of nucleic acid specific stains.

We found a pattern in increase in the total number of plaque-forming cells (PFC) in the spleen following primary antigenic stimulus very similar to that originally published by Jerne, Nordin, and Henry.^{1/} Figure 2 shows numbers of PFC in the entire spleen, plotted on a logarithmic scale, vs. the time after antigenic challenge. There is 1,000-fold increase in the numbers of PFC from the prechallenge level to the peak on day 4, followed by a gradual and continuing decrease toward control levels. At the height of the response, 0.1% of all nucleated spleen cells are specific PFC.

The most rapid increase in numbers of PFC occurs between days 2 and 3. There is a 70-fold increase during this 24-hr period. This represents a doubling time of 3.9 hr.

Figure 3 illustrates that the drastic increase in the numbers of PFC during the immune response is not accompanied by any significant change in the total number of nucleated spleen cells, about 80% of which are lymphoid cells.

Jerne,^{2/} using essentially the same system we did, estimated the maximum doubling time of PFC at 7 hr. Baker and Landy^{3/} estimated the maximum doubling time of specific antibody-producing cells during the primary response at 5.4-6.1 hr using a different antigen and a different method for identifying the immunocompetent cells.

Any of these rates of increase is too rapid to explain by ordinary cell division. Malaviya and Tennenberg^{4/} recently determined the generation time for PFC to be 13 hr, the same as for nonantibody-forming lymphoid cells.

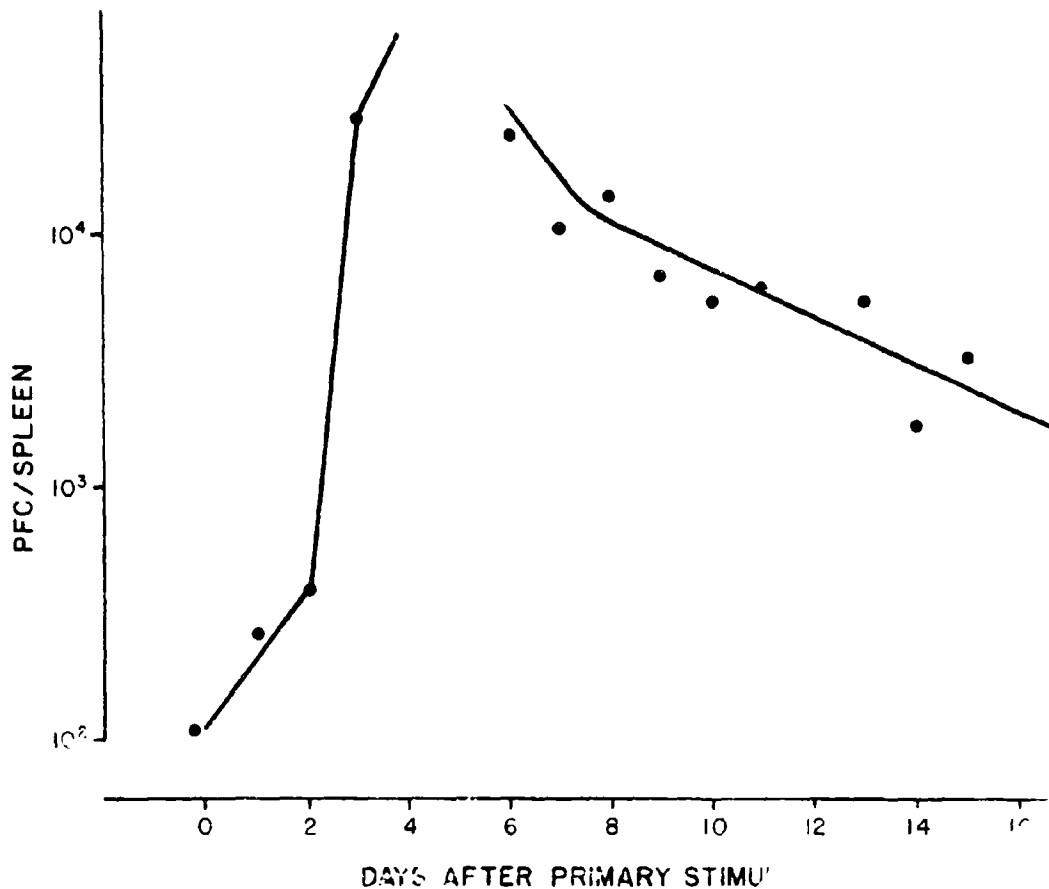


FIGURE 2. EFFECT OF SHEEP RBC ON NUMBERS OF PLAQUE-FORMING CELLS IN MOUSE SPLEEN (4 MICE / POINT).

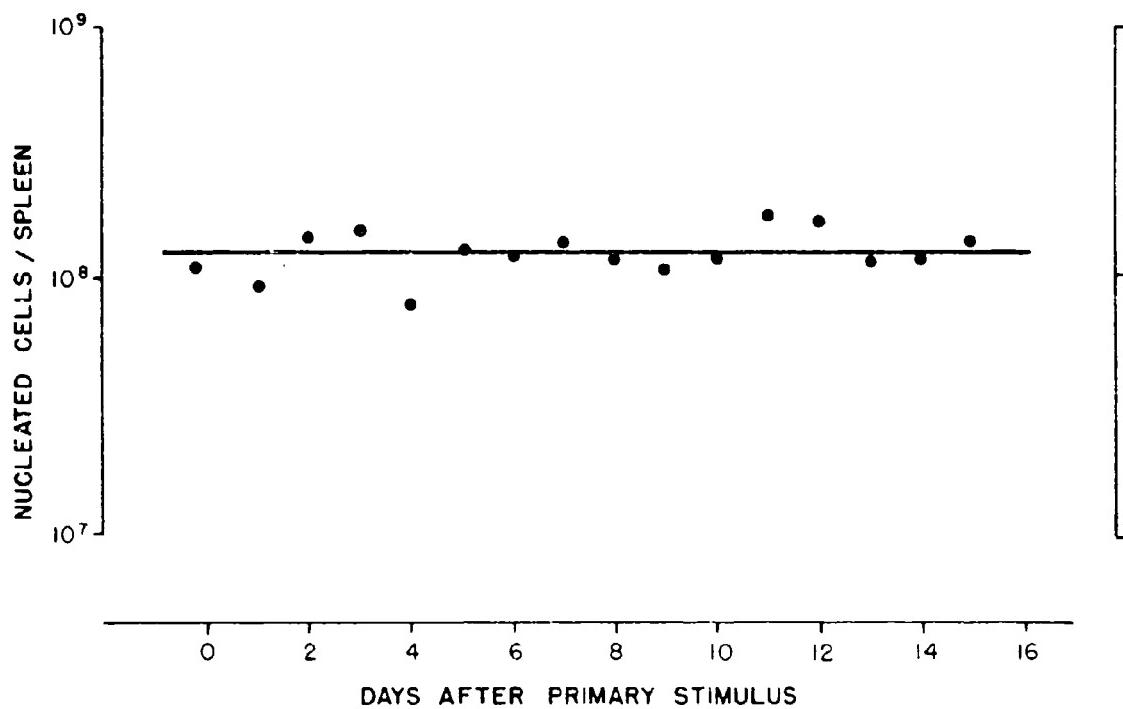


FIGURE 3. EFFECT OF SHEEP RBC ON NUMBERS OF NUCLEATED SPLEEN CELLS (4 MICE/POINT).

It remains an open question as to where the greatly increased numbers of PFC come from, if not from cell division. There is independent evidence from the work of groups headed by Friedman^{5/} and Braun^{6/} that at the height of the immune response PFC do occur in large clones. If cell division is excluded, this suggests a transfer of genetic information concerning antibody synthesis by physical contact between adjacent cells, in a manner analogous to episome transfer between mating bacteria.

We have also studied the appearance of PFC in peripheral blood following primary antigenic stimulus. In these experiments, after anesthesia with chloroform the mice were bled out and plaque preparations were made from the buffy coat cells. Figure 4 shows that there is a definite increase in the numbers of PFC in blood following antigenic challenge. In contrast to the splenic response the absolute (and relative) numbers of PFC remain quite low and there is no evidence of a sharp peak on day 4. Also, the blood response showed greater variability from one mouse to another.

To study DNA synthesis by immunocompetent cells during the primary immune response, each mouse was given 1 hr before sacrifice an IV injection of tritiated thymidine, a specific precursor for DNA. Plaque preparations were made on microscope slides from the spleens of these mice. After drying and fixation the slides were dipped in photographic emulsion, stored for 20-60 days in the dark, then developed and stained with nuclear fast red. Figure 5 shows negative and positive results. Silver grains appear at the sites of radioactive decay (arrow) and the number of silver grains around each PFC was counted as a semiquantitative measure of thymidine incorporation by that cell and therefore of DNA synthesis by that cell. Cells with ≥ 4 grains were counted as positive for DNA synthesis and cells with ≤ 3 were counted as negative. Most negative cells had none or only 1 grain while most positives had considerably more than 4 grains. Enzymatic treatment of slides with deoxyribonuclease before autoradiography eliminated the occurrence of positive cells, indicating that nearly all of the tritiated thymidine was actually incorporated into DNA.

The results of an autoradiographic study of DNA synthesis by PFC are shown in Figure 6. The fraction of PFC synthesizing DNA is plotted on a linear scale against the time after antigenic challenge. This fraction remains at 0 through the 2nd day after stimulation (although the data before day 2 is statistically inadequate). The fraction of PFC synthesizing DNA jumps to about 35% on the 3rd day, remains at this plateau through the 7th day, then gradually declines to 0. During this entire response, no more than 2% of the nonplaque forming nucleated spleen cells ever showed significant DNA synthesis.

The significance of these results is not yet clear. No reports have been published of precisely comparable experiments. However, Makela and Nossal^{7/} have studied DNA synthesis by individual antibody-producing cells during the secondary immune response, combining autoradiography with a micromanipulation technique for cellular identification. They found a

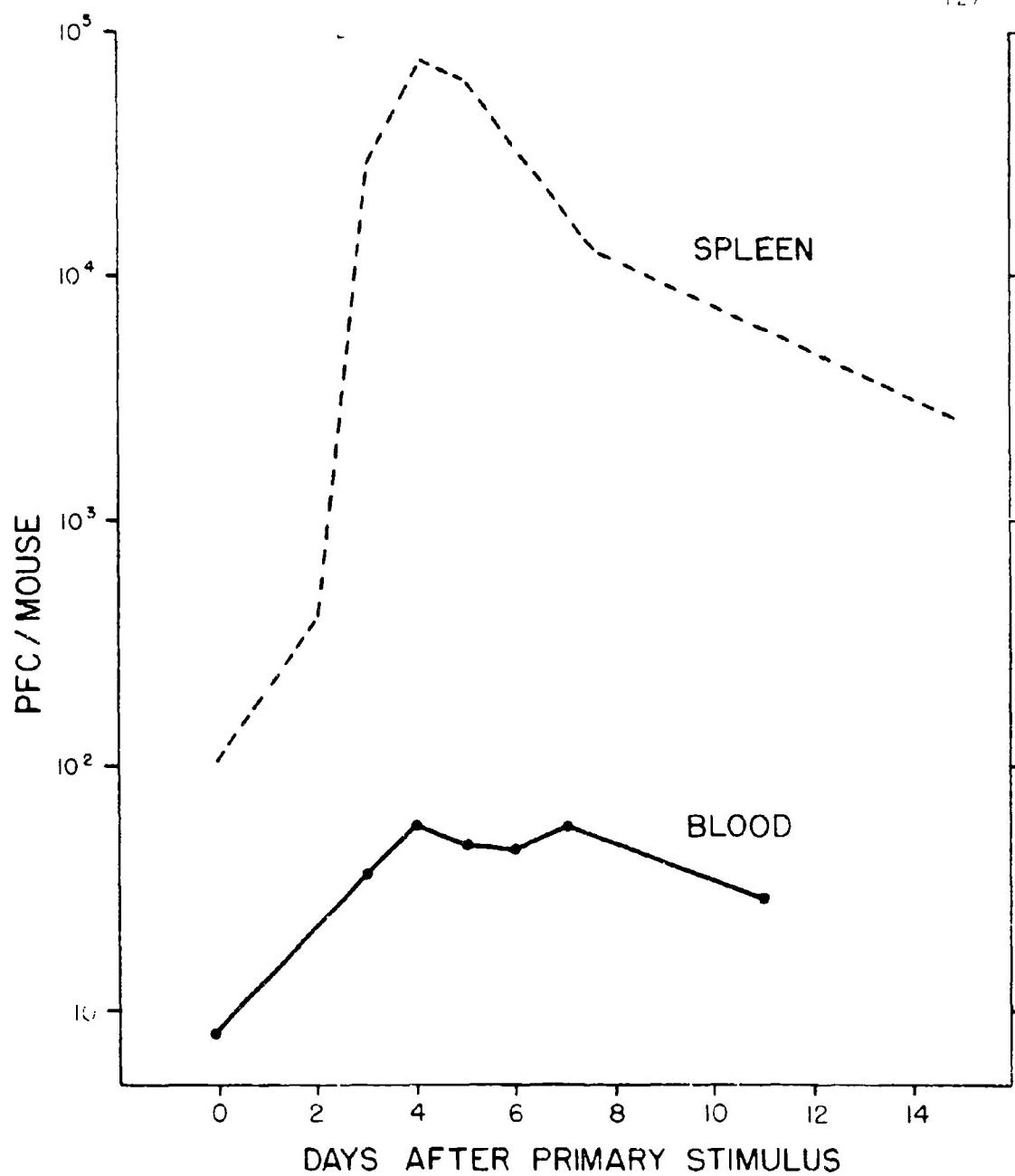


FIGURE 4. EFFECT OF SHEEP RBC ON NUMBERS OF PLAQUE-FORMING CELLS (PFC) IN SPLEEN & BLOOD (4 MICE/POINT).

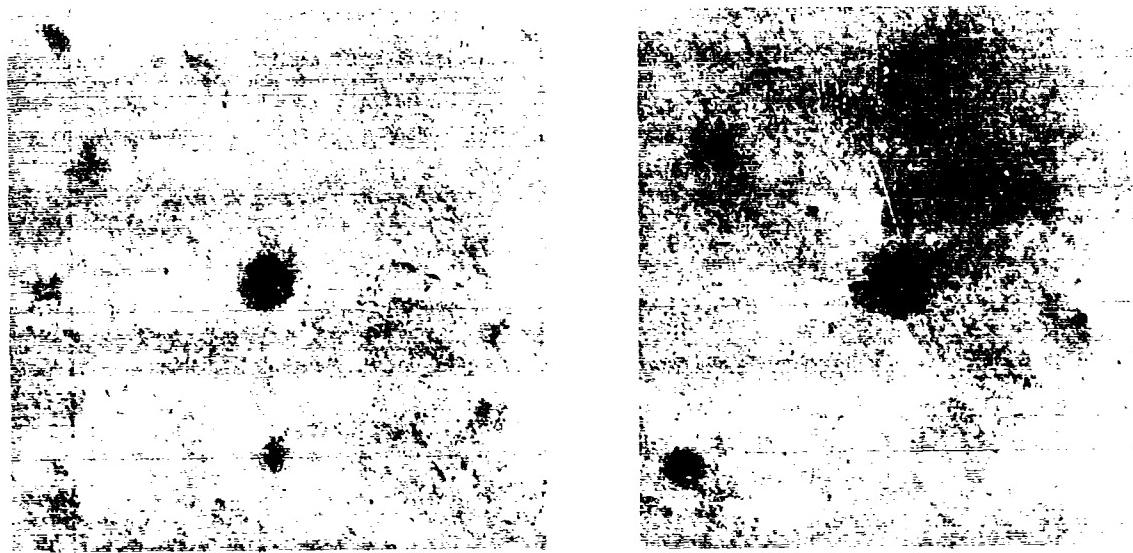


FIGURE 5. AUTORADIOGRAPHS OF PLAQUE-FORMING CELLS FROM MOUSE SPLEEN PREPARATIONS. LEFT: Ag GRAINS LOCATED ABOUT THE CELL. RIGHT: NO Ag GRAINS ARE SEEN. (NUCLEAR-FAST RED, 540X).

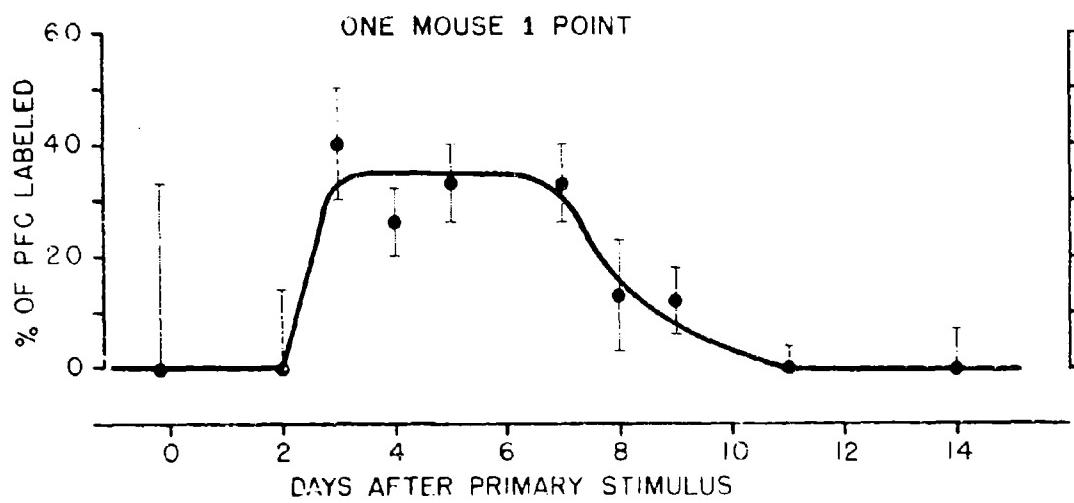


FIGURE 6. EFFECT OF SHEEP RBC ON DNA SYNTHESIS BY PLAQUE-FORMING CELLS AS MEASURED BY AUTORADIOGRAPHY (\pm SE)(1 MOUSE/POINT).

pattern of changes in DNA similar to ours, but more compressed in time, so that the fraction of DNA-synthesizing cells was 0 by day 5.

The last experiments to be described involve a study of the RNA content of individual PFC by means of staining techniques; methyl green-pyronin and acridine orange staining have been used. Like the autoradiographic technique, these staining procedures allow comparison of antibody-producing cells with other spleen cells on the same slide, since all cells have received identical treatment.

In general, DNA is stained by methyl green and RNA by pyronin, so that structures containing DNA are stained green while RNA is stained red. The color of nuclei is usually some mixture of red and green. Cells stained with acridine orange show strong fluorescent properties under intense ultraviolet (UV) illumination. DNA emits a strong yellow-green fluorescence while RNA shows a flame red color.

Using these 2 stains we attempted to distinguish RNA-positive cells from negative cells and to estimate roughly the intensity of staining in individual positive cells. Figure 7 shows in black and white various RNA-positive PFC stained with acridine orange. The RBC are not visible in these slides because they do not fluoresce. Plaques are located under white light and UV light is then used to study the fluorescence of the central cell. The cell in C looks like a large lymphocyte with a narrow ring of intensely staining cytoplasm. It was found that no more than 10% of the entire population of nucleated spleen cells ever show positive staining. PFC also show consistently more intense staining than do other cells which are RNA-positive on the same slide. At least a partial explanation of this finding presumably lies in the great increase in the number of ribosomes known to occur in PFC as shown by the observations of Hummeler et al.⁸ with the electron microscope. These ribosomes are essential to the synthesis of large quantities of antibody protein and are rich in RNA.

The spleen cell population as a whole also shows a striking response to antigenic stimulation, as measured by these stains. Both stains indicate a 10-fold increase within 48 hr of antigenic stimulus in the number of RNA-rich nucleated cells in the spleen compared with unstimulated control mice (Figure 8). This increase persists for at least a week. With acridine orange staining there was somewhat greater variability from mouse to mouse, but the findings were essentially the same.

The occurrence of large numbers of pyroninophilic cells in the spleen during the immune response has been previously observed and most investigators believe them to be precursors of antibody-producing cells. However, the absolute numbers of these RNA-positive cells in our experiments are very much greater than the maximum numbers of specific PFC which we can detect at the height of the immune response: 10% of the total number of nucleated spleen cells compared with only 0.1% for all PFC on day 4.

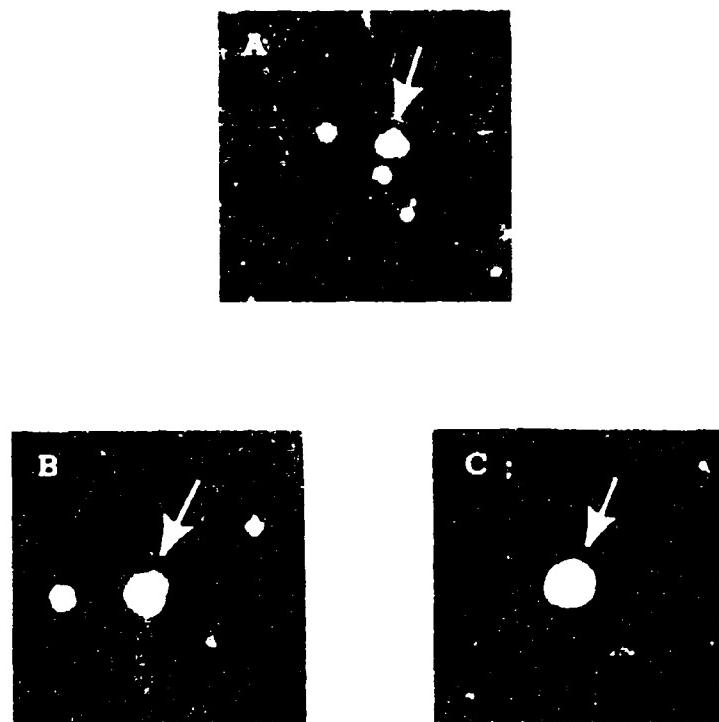


FIGURE 7. ANTIBODY PLAQUE PREPARATIONS FROM MOUSE SPLEEN CELLS. PFC ARE CENTERED. (ACRIDINE ORANGE STAIN, ULTRAVIOLET, A:250X, B,C:400X).

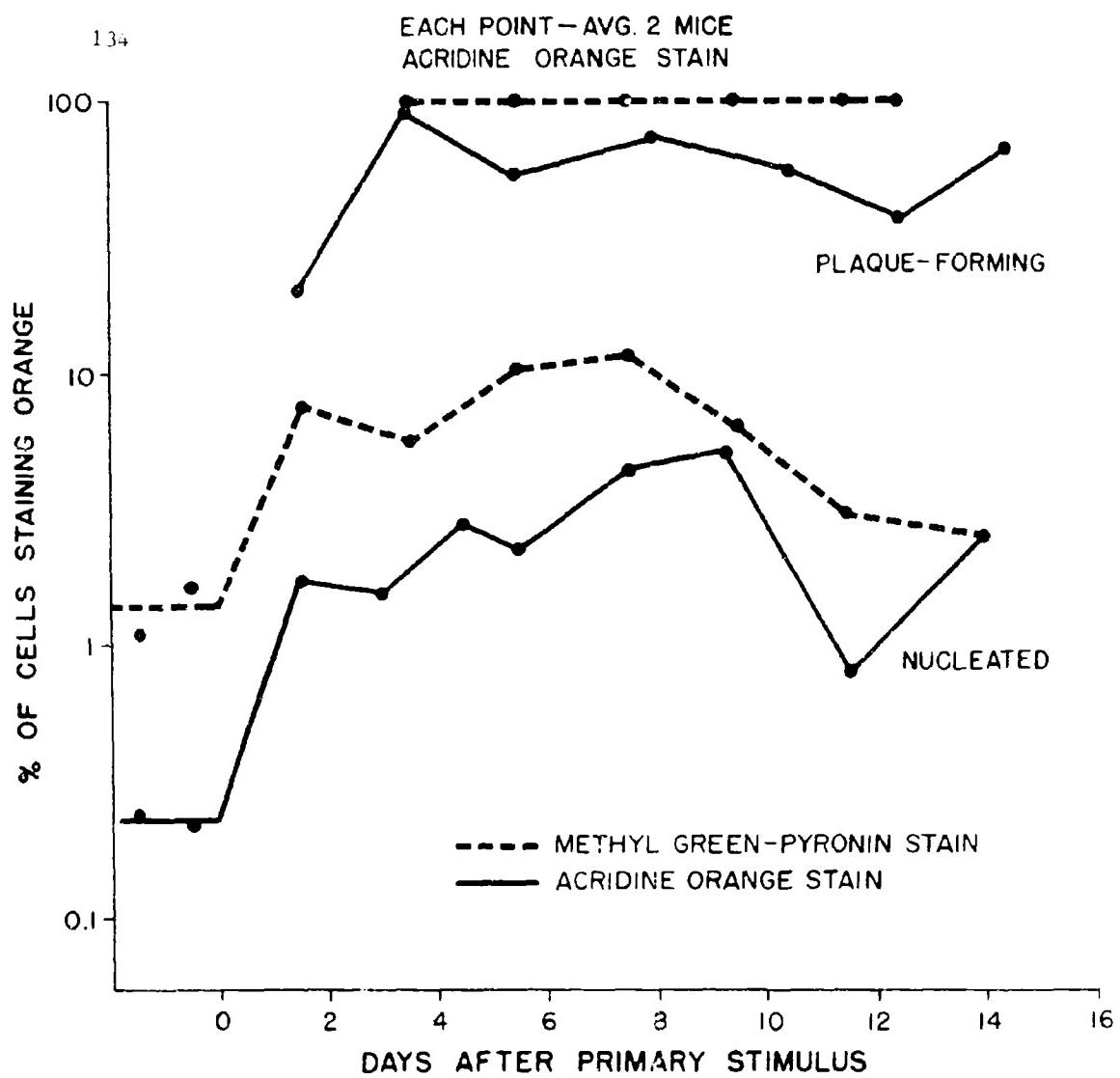


FIGURE 8. EFFECT OF SHEEP RBC ON INCIDENCE OF RNA-
POSITIVE SPLEEN CELLS (2 MICE/POINT).

What the precise relationship is between these 2 types of cells awaits further investigation. Our similar results with acridine orange staining show that the finding is not a peculiarity of the pyronine stain.

SUMMARY

The Jerne antibody plaque technique^{1/} has been adapted to produce permanent cell preparations on microscope slides. We have confirmed Jerne's results concerning the splenic response of the mouse to primary IV immunization with sheep RBC. PFC in the blood show a similar but much weaker response. DNA synthesis by antibody-producing spleen cells during primary response, as measured by autoradiography, shows a systematic pattern of change with time, which is similar to, but more prolonged than, the pattern^{2/} of DNA synthesis reported for the secondary response by Makela and Nossal.^{2/} Using stains specific for nucleic acids published findings were confirmed that there is a marked and persistent increase in the fraction of RNA-rich cells in the spleen cell population during the primary response. In addition, while there are too few PFC to explain this response, the fraction of RNA-rich cells among PFC is consistently much higher than for non-PFC.

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EARLY DETECTION OF CIRCULATING ANTIGEN

Captain Martha K. Ward, USPHS*

Some very excellent work, much of which has been done by the investigators presented in this report, has demonstrated that a number of changes in host metabolism and physiology may occur very early after infection; however, at present, none of these changes appear to be related specifically to the microorganism involved. Ideally, of course, it would be highly desirable to be able to make specific etiologic diagnosis during the incubation period. In many cases a major limiting factor is simply the very low concentration of organisms present in readily available specimens prior to and at the time of onset of clinical illness. However, it is reasonable to assume from what is known of the course of certain infections in laboratory animals, that the total number of organisms present in the body of the host at time of appearance of clinical illness is most probably relatively large. There is evidence to suggest that the first appearance of organisms in blood, sputum, and other such specimens may represent a "spill over" from larger numbers sequestered in spleen, liver, lymph nodes, or other organs. It therefore seemed possible that specific soluble antigens or products resulting from metabolic activities of the total population in the host, might be present in blood or other body fluids prior to the appearance of consistently detectable numbers of microbes.

We have recently initiated studies to determine the feasibility of detecting specific soluble antigens of bacterial agents early in the course of infection. Dochez and Avery in 1917¹ reported the presence of specific polysaccharide in the blood and urine of lobar pneumonia patients as early as the first day of illness. The method used for detection was a precipitin technique which appears to be less sensitive than some more recently developed immunological procedures. Foshay in 1936² described an organism-specific reaction to intradermal injection of hyperimmune antiserum. This immediate hypersensitivity type of reaction occurred in patients with tularemia on the first day of clinical illness. Although the skin test was rapid and apparently highly specific if the individual did not react to control serum, such foreign protein reactions did occur in some cases. The nature of the test antiserum was of special importance and, in addition, the positive reaction persisted for relatively long periods after infection, and was present in some immunized individuals.

More recent work employing a technique using latex particles sensitized with antibody for the detection of very small amounts of specific antigen suggested this approach for initial studies. Tew and Saloman³ have very recently reported the detection of as little as 0.008 µg/ml of staphylococcal enterotoxin using a microlatex agglutination test. This work was

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done with relatively purified antigen and in the absence of other proteins or body fluids. Bloomfield, Gordon and Elmendorf^{4/} used latex sensitized with Cryptococcus antibody in a slide agglutination to detect specific antigen in serum or spinal fluid of patients with cryptococcis. Antigen was detected in 7 of 9 culturally proven cases at some time during the course of illness.

For our exploratory studies, which are still in a very preliminary stage, work has been limited almost entirely to pneumococcus type I culture filtrates and infections with this organism in the Fisher 344 strain of rats. The stock culture is mouse-passed routinely every 30 days and young, actively growing, well encapsulated cultures are quick frozen and maintained at -70 C until needed. Inocula for production of culture filtrates and for animal challenge were varying dilutions of a 4-hr culture prepared in a standard fashion. Test filtrates were harvested after varying periods of incubation and the organisms removed by centrifugation and subsequent filtration through millipore filters.

In the animal studies, rats weighing 150-200 gm were challenged subcutaneously with 100-200 organisms and serially sacrificed at 8, 16, 24, 36 hr postinfection. Control animals similarly inoculated died in 48-72 hr. Samples of blood and spleen were cultured; serum was collected and frozen until use in the agglutination test.

Hyperimmune antiserum was prepared by immunization of rabbits with formalin-killed, young, capsulated organisms.

The polystyrene particles were produced by Monsanto Chemical Co. under the trade name of Lytron 615. These are 0.17-0.2 μ in diameter and the stock material contains 49 \pm 1% solids. It is known that latex particles are most stable at high pH. Oreskes and Singer^{5/} have shown that pH and ionic strength of buffer as well as the concentration of γ globulin used for sensitizing the particles markedly affect their stability and furthermore act in an interdependent fashion. The manufacturers of the particles point out that the tendency toward instability of suspensions increases with time and is affected by the kind of container used for storage. Consequently with each new system, batch of particles, and medium used, the effect of many factors must be determined and controlled.

The detailing of all technical problems we have encountered in working with latex particles would serve no useful purpose at this time. Suffice it to say, that stock particles diluted 1:600 in relatively low ionic strength borate buffer at pH 8.2 or 9.0 and sensitized with varying dilutions of hyperimmune antiserum at 56 C for 1-1½ hr have given some satisfactory preparations. A box titration with varying dilutions of culture filtrate was used to determine the concentration of antiserum giving greatest sensitivity in the agglutination test.

To select the most rapid, sensitive and reproducible procedure for performing the test, a number of possibilities were tried. Although the micro-test described by Tew and Saloman^{3/} appears to be very sensitive for their system and requires minimal amounts of reagents, overnight incubation is required for reading the test. In the procedure we are now using, 0.1 ml of sensitized particle suspension is added to an equal volume of the test sample in small test tubes. The presence or absence of agglutination is read with the aid of a concave mirror after 2 hr incubation. No significant change in titer of culture filtrates occurs at this time; with serum samples the tendency toward nonspecific reactions increases upon prolonged incubation.

The results of a box titration with varying dilutions of a 48-hr culture filtrate and particles sensitized with increasing dilutions of antiserum are shown in Table I. In this instance the preparation with a 1:1750 dilution of antiserum gave a positive reaction with the highest dilution of culture filtrate. Although not indicated here there is a suggestion of a prozone

TABLE I. TITRATION OF ANTISERUM FOR LATEX PARTICLE SENSITIZATION

ANTISERUM DILUTION (Reciprocal)	REACTIVITY ^a /								CONTROLS	
	Log ₁₀ dilutions of culture filtrate								Medium	Buffer
	-1	-2	-3	-4	-5	-6	-7	-8		
500	+	+	±	-	-	-	-	-	-	-
750	+	+	+	±	-	-	-	-	-	-
1000	+	+	+	±	±	-	-	-	-	-
1250	+	+	+	±	±	-	-	-	-	-
1500	+	+	+	+	±	±	-	-	-	-
1750	+	+	+	+	+	+	±	-	-	-
2000	+	+	+	+	+	±	-	-	-	-
2250	+	+	+	+	+	±	-	-	±	±
2500	+	+	+	+	±	-	-	-	±	±

a. Reaction of sensitized particles with incubated medium alone: -
Reaction of filtrate with normal rabbit serum particles: -

type reaction, i.e. less complete agglutination occurs in lowest dilutions of filtrate. As indicated by the \pm reactions recorded for the controls of the last two preparations in the table, particles sensitized with high dilutions of immune serum have a greater tendency toward instability and autoagglutination than those prepared with more concentrated serum. This does not seem to be the case with particles sensitized with varying dilutions of serum collected from the same rabbits prior to immunization. Table II shows the reaction of serial serum specimens from infected rats with latex particles sensitized with pneumococcus antiserum and with normal rabbit serum. No agglutination of either particle preparation occurred with

TABLE II. AGGLUTINATION^a OF SENSITIZED LATEX PARTICLES BY SERIAL SERUM SPECIMENS FROM RATS INFECTED WITH 100-200 DIPLOCOCCUS, PNEUMONIAE, TYPE I

TIME POSTINFECTION hr	REACTIVITY OF PARTICLES	
	Pneumococcus Antiserum	Normal Rabbit Serum
Preinfection	-	-
8	-	-
16	-	-
24	+	-
36	+	+
48	+	+

a. Particles in pH 9.0 borate - saline buffer.
Incubation at room temperature 2 hr.

specimens taken prior to infection or with the 8- and 16-hr postchallenge sera. The 24-hr serum specimens agglutinated the antipneumococcus sensitized particles but not those sensitized with normal rabbit serum. Pneumococci were not detected in the blood samples cultured at this time period, although spleen cultures were positive. There was a positive reaction with both normal and hyperimmune serum preparations at 36 and 48 hr. The gross appearance of the reaction, however, was different: agglutination of particles with the immune serum was largely granular in nature and resembled that seen with culture filtrates, while that with the normal sera was floccular and suggested a nonspecific reaction perhaps due to species interaction of certain serum components. It was interesting that this reaction was observed only in the 36- and 48-hr specimens. This experiment was done 2 months ago, and at the time, the results were quite

reproducible. However, in the recent reexamination of these specimens in parallel with a similar series from another group of rats, reactions with normal as well as immune serum particles were observed with all specimens when the tests were incubated at either room temperature or 37 but not at 4 C. The results of an experiment to examine the effect of temperature of incubation on agglutination of particles in both pH 8.2 and 9.0 buffers are shown in Table III. All reactions with particles sensitized with normal

TABLE III. EFFECT OF TEMPERATURE AND pH OF BUFFER ON AGGLUTINATION OF SENSITIZED LATEX PARTICLES BY RAT SERA PER- AND POSTINFEC-

		REACTIVITY OF PARTICLES							
TIME POSTINFECTION	hr	Pneumococcus Antiserum				Normal Rabbit Serum			
		pH 8.2	pH 9.0	pH 8.2	pH 9.0	pH 8.2	pH 9.0	pH 8.2	pH 9.0
		37 C	4 C	37 C	4 C	37 C	4 C	37 C	4 C
Preinfection		+	±	+	-	+	-	+	-
	8	+	±	+	-	+	-	+	-
	16	+	±	+	-	+	-	+	-
	24	+	+	+	+	+	-	+	-
	36	+	+	+	+	+	-	+	-

rabbit serum are eliminated by incubation of the tests at 4 C; what we are considering to be specific reactions with antiserum particles do not appear to be affected. Particles sensitized with specific serum in pH 8.2 buffer had some tendency toward autoagglutination even at 4 C as indicated by the ± reactions in the 3rd column. However this is not the case with pH 9.0 preparations. If we allow ourselves to ignore part of the table and look only at tests with pH 9.0 buffer incubated at 4 C we see the same results obtained in the first experiment with immune serum; in addition the reaction with normal rabbit serum seen originally in the 36- and 48-hr postinfection specimens has been eliminated.

The only explanation we have at the moment for failure to reproduce the results of the initial experiment with tests incubated at room temperature or 37 C is that the increased age of the stock of latex may be responsible for increasing instability of the particles.

It is at least somewhat encouraging to have demonstrated what appears to be specific antigen or antigens in the serum of animals prior to the time of consistently demonstrable bacteremia. However it is obvious that more basic studies at every step of the procedure with sensitized latex particles are needed. We have not yet determined quantitatively the ultimate sensitivity of the technique. We have looked at only one infection in a single host. It may be that the usefulness of this approach will be limited both by technical difficulties and lack of general applicability to other infections or other organism-host systems.

On the other hand, in view of the theoretical possibilities and the albeit very limited encouragement from these preliminary studies, we feel that some further work is justified.

The excellent technical assistance of Mr. Ralph G. Knodel, Mr. Wallace Fee and Mr. Howard Cole is gratefully acknowledged.

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EARLY CHANGES IN GAMMA GLOBULINS

Virginia G. McGann, Ph.D.*

The present studies were initiated recently to determine whether differential changes in intravascular levels of human immune γ G-, γ M- and γ A-globulins (IgG, IgM, IgA) could be detected in the early stages of infection or after immunization. They were one aspect of the broad study of metabolic and biochemical responses of healthy volunteers to immunization and to experimental disease. Investigations are in a preliminary stage; data are available from only a single experiment in which healthy adult male volunteers were immunized with the 17-D strain of yellow fever virus.

Well documented studies of human response to the 17-D strain indicate the presence of many factors that might affect γ -globulin levels. Sweet et al.¹⁷ found viremias in approximately 60% of subjects after primary exposure, but not after secondary exposure. Neutralizing antibodies appeared as early as day 7 with maximum titers at days 21-28. Wheelock²⁷ demonstrated the temporal relationship of viremia, interferon and antibody. Peak amounts of interferon were present within 24 hr of maximum viremia (days 5-7), and hemagglutination inhibiting (HI) antibody appeared within 24 hr after peak interferon levels.

Viral neutralizing antibodies have been associated with IgG, IgM and IgA globulins, the location depending on time after exposure, the nature of the lesions and the viral agent. The earliest antibody response appears within 7 days in the IgM fraction. IgM antibody is replaced by IgG at about 14 days; and in herpes-type infections, a further shift to the IgA fraction occurs at about 21 days. Quantitation of human γ -globulins after infection has been limited to study of occasional samples obtained after hospitalization, and measured values have compared with the means for the normal population. Because of the spread of values for a healthy adult population, however, only extreme changes could be detected. Deviations from population means have been reported chiefly for chronic bacterial infections, or in conditions where local lesions are involved. Few viral diseases altered γ -globulin levels significantly. Volunteer studies offered a unique opportunity to investigate the effect of a mild virus infection where postexposure measurements could be related to mean pre-exposure values for each individual.

Ten volunteers participated in the study. Eight individuals received the standard immunizing dose of 0.5 ml of 17-D vaccine, containing 10^6 median mouse intracerebral lethal doses. Four had no known prior experience with the vaccine; the log neutralization index (LNI) of their pre-exposure sera was < 1. These individuals constituted the primary exposure group. Two previously immunized and 2 with no known prior experience had

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preexposure neutralizing activities equivalent to that of immunized volunteers, LNI \geq 3. These individuals constituted the secondary exposure group. The remaining 2 individuals were used as controls and received an injection of physiological saline instead of vaccine.

Plasma samples were obtained from each subject at 12-hr intervals and stored at -20°C until use. Sampling was initiated at 0800 hours, 24 hr before inoculation, and continued for 14 days. The 3 globulins in every sample were quantitated by the single radial diffusion method of Fahey and McKelvey.³⁷ Slides with single factor antiserum incorporated in the agar were obtained from Hyland Laboratories. All samples from an individual were tested at the same time. Experimental error of the procedure was \pm 10% between days and < 5% for tests run on the same day. Differences between measurements of preexposure bleedings were within the experimental error, and arithmetical means of values for these bleedings were considered to represent normal values for each individual (Table I).

TABLE I. MEAN PREEEXPOSURE VALUES FOR IgG, IgM, and IgA GLOBULINS OF INDIVIDUALS IN THE CONTROL GROUP AND IN THE PRIMARY AND SECONDARY EXPOSURE GROUPS

GROUP	SUBJECT	PREEEXPOSURE MEAN (mg/100 ml)		
		IgG	IgM	IgA
Control	1	920	65	60
	2	1590	65	680
Primary Exposure	1	955	45	80
	2	1150	85	170
	3	1070	25	205
	4	850	55	80
Secondary Exposure	1	845	95	211
	2	1130	50	360
	3	1470	55	450
	4	980	80	320
		843-1349	42-82	66-456
Mean \pm 1 SD				
McKelvey and Fahey ^{4/}		1020-1460	85-155	210-350
Stiehm and Fudenberg ^{5/}		975-1450	70-125	140-250

The range of values for IgG was 845-1590 mg/100 ml, for IgM, 25-95, and for IgA, 60-680. A number of the values, particularly for IgM, fell outside the normal range for adults.^{4,5} Further study revealed that frozen plasma was unsatisfactory for IgM determinations, probably because it coprecipitated with fibrogen. Parallel tests of serum and plasma indicated that although IgM decreased in frozen plasmas, the relationship between values for serial bleedings of the same individual was not altered. The use of plasma did not appear to affect determinations of the other 2 globulins.

Daily variations in globulins for the control individuals are shown in Figure 1. Time is recorded as days pre- or postinoculation. Values for samples obtained at 0800 and 2000 hours gave no evidence of a circadian rhythm for γ -globulins. IgG appeared to have a cyclic response with a 3-day periodicity. By days 2 or 3, values for both individuals showed an increase of about 40% above the preinjection value; lower peaks occurred on days 6, 9, and 10. The IgM response apparently followed a similar course. Levels of IgA globulin showed significant decreases; Subject 1 had a low preinoculation value which decreased about 60% almost immediately; that of Subject 2 with a very high control value decreased more slowly by about 40%. The stress of chronic blood loss, a total of approximately 750 ml, may be responsible for these changes in the control subjects. In a more recent study when approximately half this volume of blood was lost in daily bleedings, control subjects showed a similar pattern of response but deviations from the mean were only about 20%.

Although the controls showed characteristic patterns of response, the same could not be said for immunized subjects in which the stress of immunization was superimposed on the stress of bleeding. Individual variation within each exposure group was so great that the response of each subject is recorded (Figure 2). Individuals are presented in the order of decreasing liver involvement as indicated by serum transaminase and bilirubin measurements. The first 3 subjects had significant involvement, and the 4th, none. No viremia was detected after immunization but all subjects developed neutralizing antibodies by day 28 with LNI ≥ 3 .

The additional stress of immunization had its greatest effect on IgG and IgA responses. None of the group had an IgG response like the controls. Subject 1 showed marked diurnal variation from + 60% in the evening to -75% in the morning between days 1 and 6, the time interval in which viremia might be expected. After day 6, it was elevated. The other 3 individuals had levels that were not markedly different from their means, although there may have been some suppression. Three in the primary exposure group had significant elevations in IgA, and the 4th, Subject 3, showed an initial depression, followed by partial recovery at day 4. Except for Subject 1, IgM response was not significantly different from that of the controls.

Response patterns for the 3 globulins after secondary exposure are shown in Figure 3. All individuals had preexposure LNI ≥ 3 ; none developed viremia. Subjects 1 and 3 had prior immunization with 17-D vaccine;

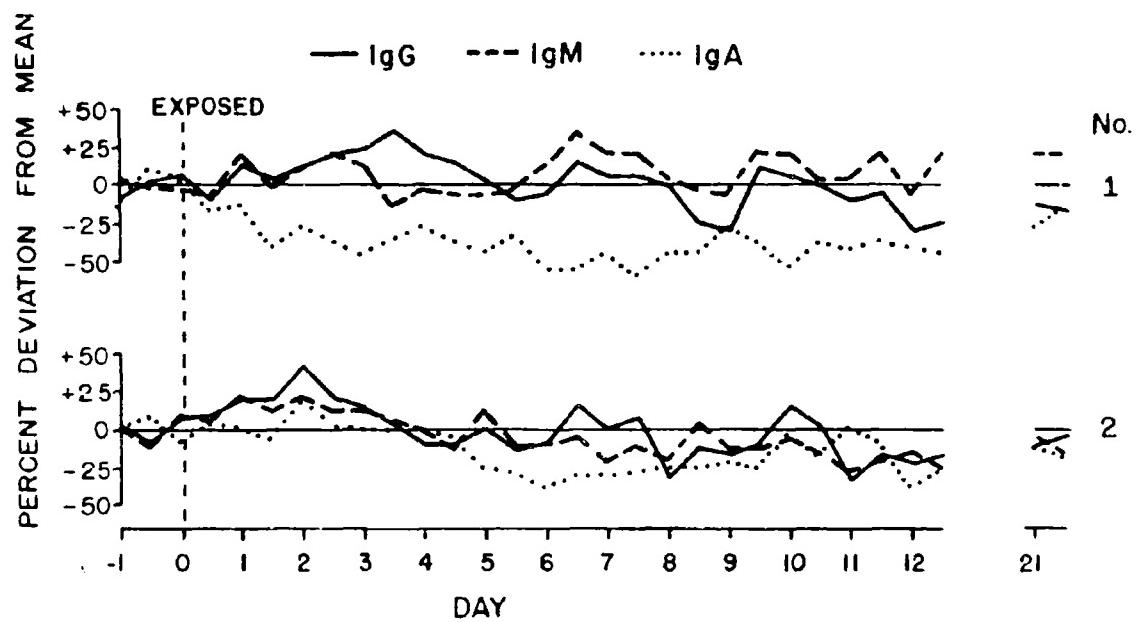


FIGURE I. IMMUNOGLOBULINS IN NONIMMUNIZED SUBJECTS.

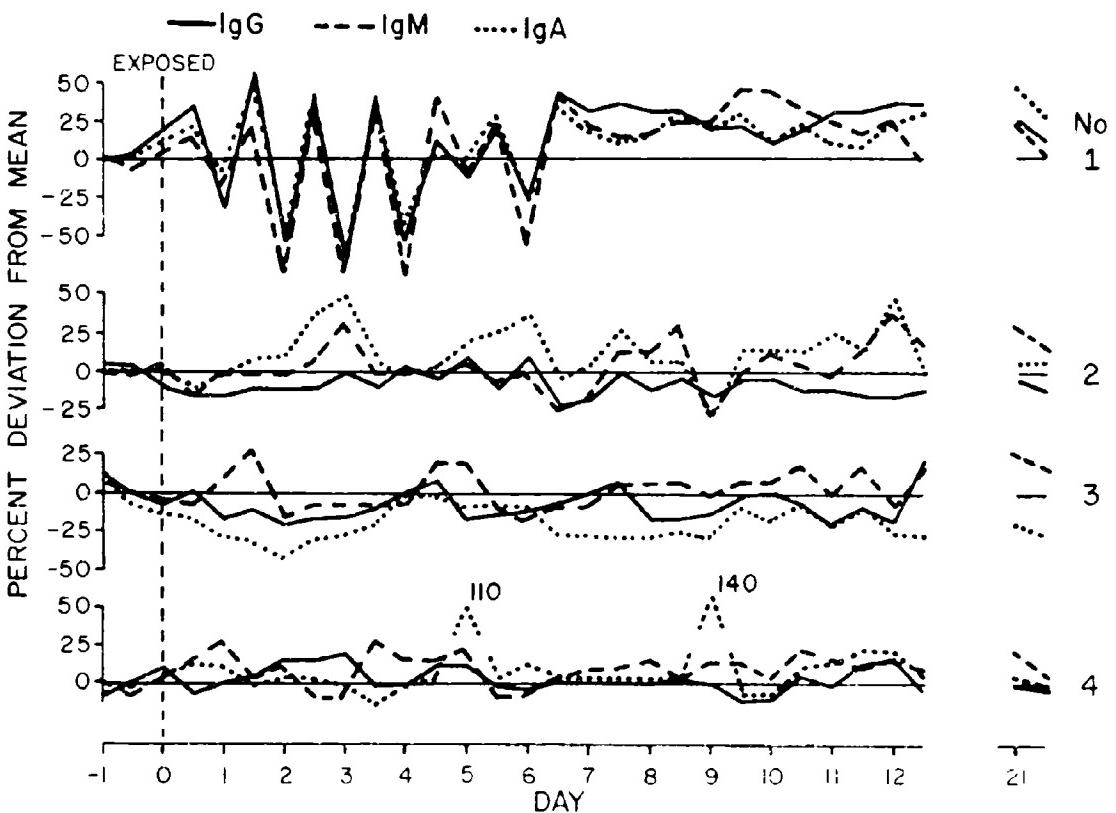


FIGURE 2. IMMUNOGLOBULINS AFTER PRIMARY IMMUNIZATION WITH 17D YELLOW FEVER VACCINE.

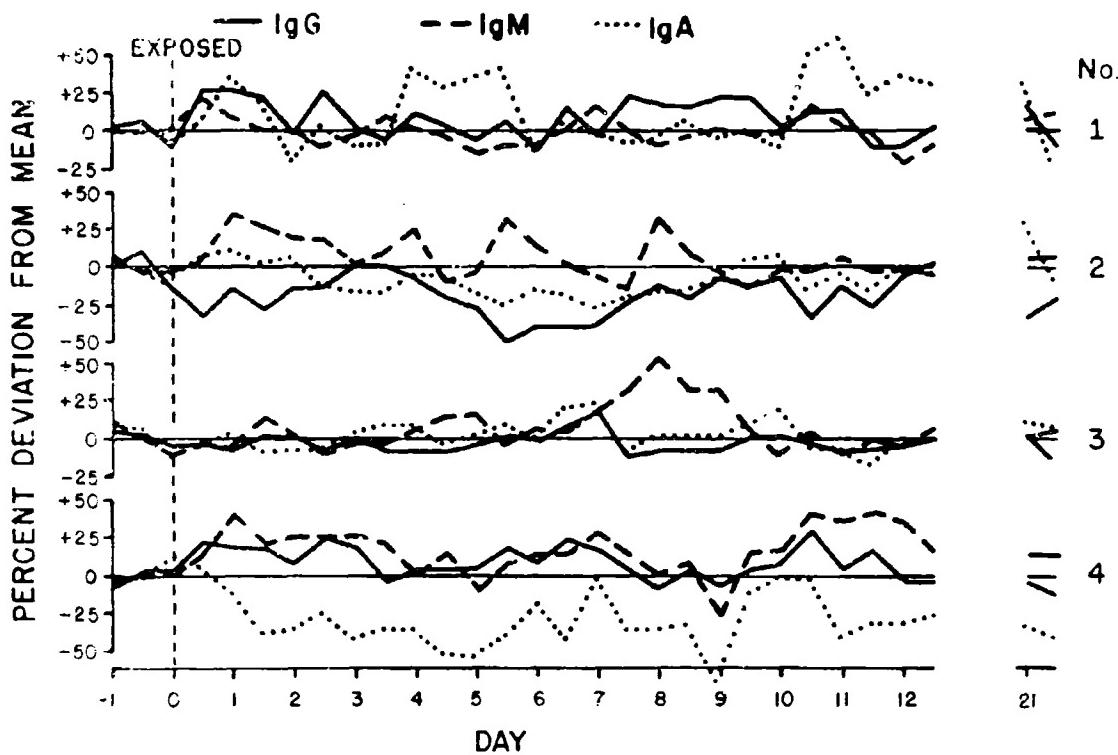


FIGURE 3. IMMUNOGLOBULINS AFTER SECONDARY IMMUNIZATION WITH 17D YELLOW FEVER VACCINE.

Subjects 2 and 4 had no previous history of immunization or exposure. Although it might be expected that the presence of neutralizing antibody would localize the infection and limit liver involvement, the first 3 individuals had significant elevations in serum transaminase on day 7. As was true of the primary exposure group, marked individual variation was seen in IgG and IgA responses. The former was elevated in one individual, suppressed in the 2nd, remained close to the preexposure mean in the 3rd, and, in the 4th, followed a pattern similar to that of the controls. The IgA response was more variable than in the primary exposure group. The first subject had significant elevations; the 2nd showed suppression, with partial recovery on days 4 and 10; the 3rd had no significant change and the 4th responded like the controls. The IgM response was not significantly different from that of the controls except in Subject 3, who showed an increase of approximately 60% on day 8.

Subject 4 in the secondary exposure group was the only 1 of 8 immunized individuals to have an overall pattern of response like that of the controls. In all other vaccinees the cyclic behavior of IgG was modified or suppressed; and in most vaccinees no IgA suppression occurred. When IgA decreased, there was partial recovery by day 9.

The small number of individuals and the variation within each group make it difficult to define and compare group responses. Arithmetic means of deviations from preexposure values and standard deviations for these were calculated for the 3 experimental groups at each bleeding time. There was considerably more variation within the immune groups than within the control group, and the standard deviation around each point after primary exposure was generally greater than after secondary exposure. Group means are plotted in Figure 4. The patterns of response for the control group are probably representative for similarly treated nonimmunized individuals. The 3-day cycle of IgG and the depression of IgA are clearly seen; IgM appears to follow the IgG response. In the primary exposure group Subject 1 was responsible for diurnal variation between days 1 and 4, and as might be expected, standard deviations of values in this time interval were very high. Nevertheless, IgG and IgA responses of the primary exposure group were significantly different from those of the controls, and the elevation in IgM values after day 9 may be significant. After secondary exposure, average values for all fell between those of the control and primary exposure groups.

Individual variation and small-number sampling make it hazardous to speculate on factors responsible for early changes in intravascular levels of γ -globulins. Nonspecific response to the stress of bleeding or to secondary effects of tissue damage may be involved. The minor stress of daily bleeding appeared to have a marked effect on the control group, and the high degree of individual variation between vaccinees may suggest a reaction to stress rather than to a specific viral agent.

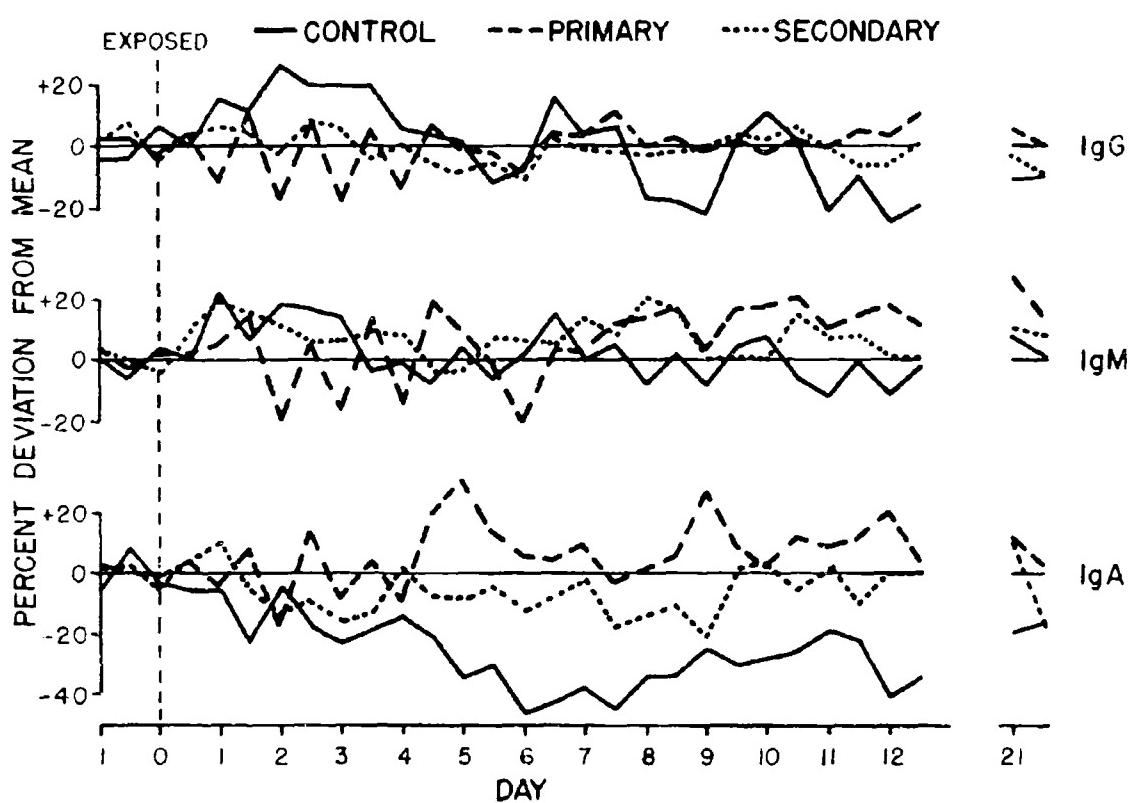


FIGURE 4. IMMUNOGLOBULIN RESPONSE BY GROUP AVERAGES FOLLOWING MORNING EXPOSURE.

SUMMARY

Data from a preliminary study on early changes in γ -globulins after immunization with the 17-D strain yellow fever vaccine were presented. The γ -globulins in plasma samples from 2-4 volunteers were quantitated for 14 days after sham-inoculation or after primary or secondary immunization. The patterns of IgG, IgM and IgA were more consistent in sham-inoculated individuals than in vaccinees, and the response of the controls suggested that the stress of bleeding may have a significant effect on γ -globulin levels. The γ -globulin responses after immunization showed a high degree of individual variation, but IgG and IgA responses were significantly different from those of control subjects. The effect of primary exposure appeared to be greater than that of secondary exposure. Further study is required to distinguish between the response to immunization or infection and the effect of nonspecific factors.

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DISCUSSION OF SECTION III

James G. Hirsch, M.D.*

To open the discussion I will make a few general comments concerning the three papers we've just heard.

When the Jerne plaque technique was developed a few years ago, it seemed likely that it could be used to answer some basic questions about antibody production. Specifically, observations on kinetics of the appearance of antibody-producing cells should resolve the question of differentiation vs. proliferation, i.e., does antigen lead to differentiation of a large number of preexisting cells to an antibody producing state, or does it lead to selection and proliferation of a single or a few cells endowed as a result of mutation with ability to produce the specific antibody. Also, examination with modern cytologic tools of the cell in the center of a plaque should resolve the long standing question of the type of cell which produces the antibody.

As to the first question of differentiation vs. proliferation, the results, including those of Dr. Krisch are inconclusive; the rate of appearance of new antibody-forming cells is too rapid for proliferation, at least in relation to the usual generation time for known rapidly growing cells, yet this progressive rapid increase is certainly suggestive of proliferation rather than simply differentiation or maturation of preexisting cells. Perhaps both processes are at work.

Now in relation to the second question on the nature of the antibody producing cell, three separate research teams have now studied by electron microscopy the cells at the center of Jerne plaques, and the results are again inconclusive because the cells in the center of the plaques are not of uniform morphology. A good many of them have all the characteristic features of plasma cells, which certainly is not surprising. Some seem to have features of small lymphocytes, and a good many of the cells in the center of the plaques have those features described by Dr. Krisch, i.e., they are rather large cells with immature nuclei and cytoplasm containing polyribosomes.

There are limitations in the Jerne technique which are important to keep in mind. We can not be sure that the cell in the center of the plaque actually is producing the antibody. It is conceivable that this cell has absorbed or ingested antibody that was produced by another cell and that it simply is releasing this antibody during the period of incubation in the agar plate or slide. Another limitation of the technique is the fact that antibodies to red cells are antibodies of a very special type, i.e., for

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the most part 19S. Some of the findings with the red cell technique may not be applicable to other systems or other types of antigenic stimulation or to other types of antibody response.

Now Dr. Ward's efforts to do something about early diagnosis using techniques for detection of antigen are certainly to be encouraged. This is an old problem. Early diagnosis of infectious disease has been a major concern of this Commission for some time. We've had conferences on it in the past. It has been a much-talked-about subject with very little progress in terms of practicable new methods. We all know that the standard methods of culture, whether for viruses or for bacteria, are inadequate to provide the physician with a rapid diagnosis at the time he needs it. In the case of severe infections, almost always some sort of educated guess work is indicated in terms of prescribing therapy. Now there were methods mentioned suggested that there may be consistent biochemical differences in the host in terms of virus vs. bacterial infections. In the case of respiratory tract infections there certainly are differences in the exfoliative cytology. This was looked into by several people many years ago and a skilled exfoliative cytologist can, in almost all instances, distinguish between viral and bacterial infections of the upper respiratory tract and do it very quickly. The difficulty here is that it is a demanding technique and just not practicable. In other words, in addition to speed we need something that is practicable, and hopefully, specific.

The introduction some years ago of fluorescent and radioactive techniques for studying antigen-antibody reactions and phenomena gave hope that application of these, in one way or another, by some technique, would answer this problem of early diagnosis. Unfortunately that has not been the case. On the whole, the use of fluorescence techniques has been disappointing in terms of practical results. Certainly we would all agree that the thing to look for is antigen if you want an early specific diagnosis. The detection of specific antigen is a tough problem. It is a problem that may be insoluble in terms of the number of antigens that one would have to look for, and in terms of the type of techniques that must be available. Nevertheless this type of study is sorely needed.

Dr. McGann's studies concerning the response of different classes of immunoglobulin to challenge with a viral infection is also a very commendable type of approach. Much progress has been made in terms of characterizing immunoglobulin classes and their primary and secondary structures and in developing methods for distinguishing between the different classes of immunoglobulins. These biochemical studies of the immunoglobulins have been extensive in recent years. However, we need much more information on the biology of the immunoglobulins. Why do we have the different classes? Do they serve different functions? Are they produced by different types of cells? Many of these fundamental aspects of immunoglobulin biology remain to be worked out, and certainly information on immunoglobulin response of the kind described is to be desired. In conducting such studies, one should remember that perhaps the blood stream is not the only place to look.

Certainly IgA is produced at cell surfaces, and its presence in the blood stream may not be an accurate reflection of what is going on at the site of it's cellular interaction. The same might be said of the enzyme and biochemical studies that were described in Section I, that perhaps the blood stream is not always the best, or at least not the only place, that one should look.

What this whole session points out, to my way of thinking, is the fact that despite the enormous advances in the field of immunology in the past few years there remain very many important central issues, both applied and fundamental issues, that need further work.

DR. GREISMAN: There is one question I would like to ask Dr. Ward. You are probably familiar with Bozicevich's use of bentonite sensitized with gamma globulin in detecting viral antigens in very small quantities. I wonder if you might have fewer problems if you used different types of particles such as bentonite?

DR. WARD: This certainly may be the case. We hope to look at it. However, the experience of one of the people working in our laboratory with bentonite for another purpose has uncovered certain difficulties with handling those particles too. I think we are going to have to do some very basic studies on the colloid chemistry of the latex particles or try to find some other way of getting at the problem of antigen detection.

DR. ZAMECNIK: Since the intracellular appearance of RNA replicase is such an unusual feature of an RNA viral infection, I wonder if it would be worth more effort to try to increase the sensitivity of methods for detecting that. Dr. August, who works in this area, has commented that detection of viral RNA synthetase might be feasible if one could step sensitivity up by a factor of 100 or more by using very highly specific radioactivity.

DR. KLAINER: With reference to a rapid differential diagnosis of bacterial vs. viral infection, I would like to call attention again to a poorly appreciated fact. A simple nasopharyngeal smear will frequently show differences between viral and bacterial upper respiratory tract infections. We did a study when I was in training concerning the polymorphonuclear leukocytes revealed by staining nasopharyngeal smears of children. Large numbers of leukocytes were indicative of bacterial infections in about 90% of the cases whereas they were absent in viral infection. In infectious mononucleosis one may see what looks like pus dripping off the tonsils and off the back of the throat, yet a smear will reveal no polys at all. This technique is practical, it is simple, and it is something that has been completely forgotten. This can be done by a medical student or a high school student.

DR. HIRSCH. I wish there were more people like you. More than 10 years ago Dr. Pierce and I with others did a study on this specific point and published the results. Almost 100% of the time one could distinguish between acute bacterial and acute viral upper respiratory infections by nasopharyngeal cytology. I have concluded that it was not practicable, because nobody ever uses the method.

DR. WOOD: A similar concept holds true for the other end of the gastrointestinal tract. Isn't that so?

DR. HIRSCH: For infection?

DR. WOOD: Yes. In differentiating bacillary dysentery from amebic dysentery, for example.

DR. HIRSCH. It works well.

DR. WOOD: I would like to thank the speakers for their splendid presentations and also the discussants. I think we've really been treated to an excellent program, a stimulating program, this afternoon and I know that all of us in the audience are grateful to the speakers and discussants.

SECTION IV

INFECTION AND GENERALIZED HOST RESPONSES

HORMONAL RESPONSES

MODERATOR: Dr. Leighton E. Cluff

DISCUSSANT: Dr. Sidney H. Ingbar

THYROID HORMONES AND INSULIN

Major George E. Shambaugh, III, MC*

The deleterious effect of a generalized infection in the diabetic or myxedematous patient is well known, but knowledge concerning the significance of insulin or peripheral thyroid hormone responses during a generalized infection is limited.^{1,2/} To gain information concerning the possible influence of these hormones, studies of alteration in carbohydrate metabolism, peripheral thyroid hormone metabolism and alterations in an easily identified enzyme protein have been undertaken in man^{3/} and the laboratory rat^{4/} during carefully controlled bacterial infections.

Alterations in carbohydrate metabolism during a generalized illness, were studied prospectively in volunteers prior to infection, during the febrile clinical illness of acute respiratory tularemia and during convalescence 2 weeks following defervescence.

In Figure 1 are shown glucose disappearance rates following a rapid intravenous (IV) infusion of 60 gm of glucose given to healthy nondiabetic males. The mean \pm 1 SE are plotted for 6 patients prior to infection, during febrile illness, and in convalescence 2 weeks following return of the temperature to normal. The slowed rate of glucose disappearance during clinical illness is suggestive of carbohydrate intolerance.

Serial concentrations of individual immunoreactive insulin responses in 7 patients following IV glucose loading are shown in Figure 2. The enhanced insulin response during maximum infection should be noted and its return in the majority of patients toward a preinfection control 2 weeks later.

An enhanced insulin response in the presence of an abnormal glucose tolerance is compatible with a peripheral inhibition of insulin action. The enhanced insulin response during fever suggests that fever might be a factor, but persistence of an enhanced insulin response 2 weeks following defervescence implicates other factors as well. Among these factors adrenocortical steroids and growth hormone may play roles. The pattern of insulin response during acute illness resembled that seen following cortisone administration. To examine this possibility more closely, we measured whole blood pyruvate concentrations following IV glucose loading (Figure 3).

It should be noted that the increased pyruvate concentrations in the clinically ill patients are suggestive of increased pyruvate production or inhibition of a peripheral utilization of pyruvate. A similar increase in whole blood pyruvate concentration has been reported in steroid diabetes.

* U. S. Army Medical Unit.

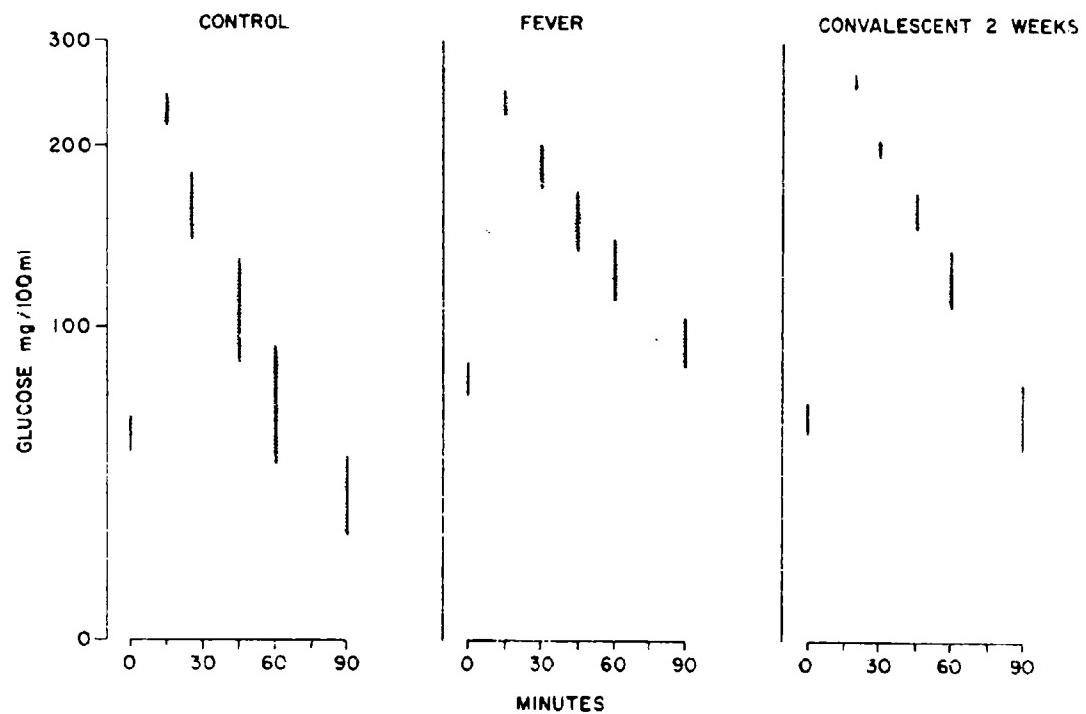


FIGURE I. DISAPPEARANCE RATES OF IV GLUCOSE IN TULAREMIA IN NONDIABETIC MEN (ISE).

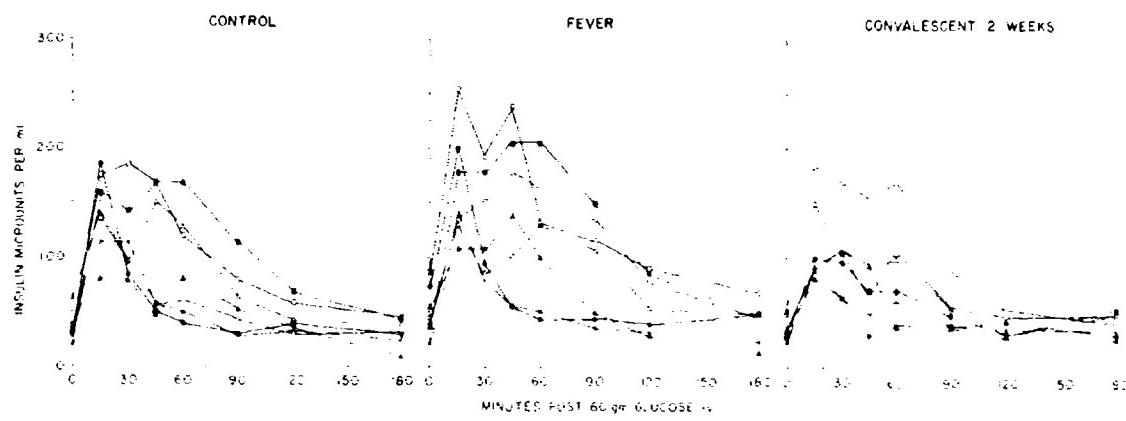


FIGURE 2. SERUM INSULIN RESPONSE TO A GLUCOSE LOAD IN 7 MEN

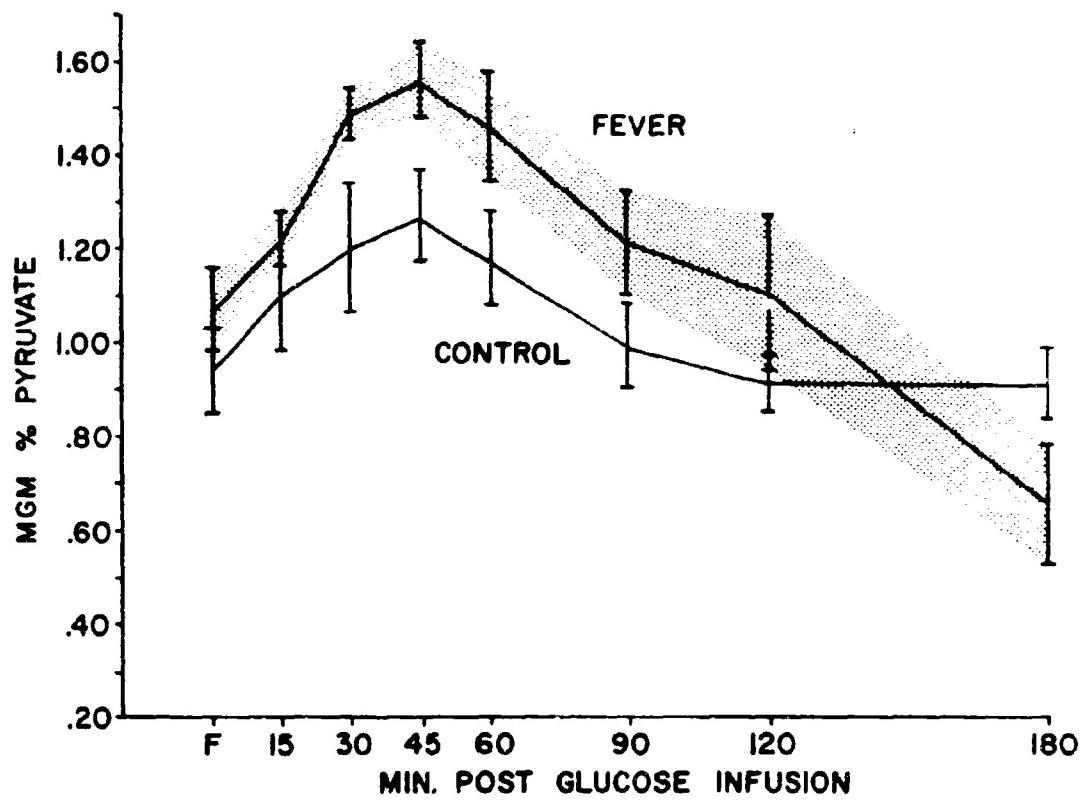


FIGURE 3. WHOLE BLOOD PYRUVATE CONCENTRATIONS DURING TULAREMIA IN MAN (\pm SE).

In insulinopyruvic diabetes, pyruvate concentrations fall, while in insulin-resistant diabetes of obesity, no change in the whole blood pyruvate has been seen following IV glucose loading.^{5/}

It would be attractive to attribute the enhancement of insulin response and peripheral inhibition of carbohydrate metabolism to adrenocorticoid elevations during acute infection, but the persistence of the abnormalities in carbohydrate metabolism in a few patients 2 weeks postinfection, when adrenocortical hormone output has returned to normal, suggested other factors. Curiously enough, abnormalities in carbohydrate metabolism paralleled in time the negative nitrogen balance seen during this infection. Nitrogen metabolism and carbohydrate metabolism may therefore be related. Tyrosine transaminase, an enzyme found largely in the liver contributes to both protein metabolism and to the production of acetoacetate. Possibly activation of transaminases producing a persistent gluconeogenesis may perpetuate insulin antagonism during convalescence. Since insulin itself contributes to synthesis of certain enzyme proteins, an investigation of insulin-dependent enzymes at the molecular level may provide information concerning the mechanisms underlying abnormalities in carbohydrate metabolism during infection.

Carbohydrate intolerance and fever during a generalized infection bear a similarity to thyrotoxicosis. In fact, Farrant in 1914^{6/} described a variable but frequent development of colloid depletion and thyroid acinar cell hyperplasmia compatible with increased thyroid gland activity in patients dying from a number of infectious diseases. A pilot study of protein bound iodine (PBI) determinations during acute febrile illness is shown in Figure 4. The mean and its 95% confidence limits shown in this group of patients reveal a progressive fall in the PBI reaching a significant depression below the control on the day of maximum fever. Since these findings appeared to be in conflict with the older literature as well as a recent report of thyroidal hyperactivity during recovery from pneumococcal pneumonia a prospective investigation of sequential alterations in peripheral thyroid hormone physiology was undertaken in volunteers following exposure to aerosolized Pasteurella tularensis. During this study, no aspirin or iodine-containing medications were given.

In Figure 5 are shown the means \pm 1 SE of the percent unbound thyroxine measured by a resin dialysis technique, the PBI, and the derived concentration of circulating unbound physiologically active thyroxine in 8 volunteers. The unbound thyroxine (PFT₄ and AFT₄) begins to rise within 24 hr of exposure, reaching a peak at the onset of fever, then returns toward normal. A similar transient change has been described by Surks and Oppenheimer^{7/} in patients following herniorrhaphy. PBI fell initially and then rose following institution of therapy and onset of recovery. The derived concentration of unbound thyroxine fell during acute febrile illness. Since every finding shown in the figure can be supported with data obtained by different observers from groups of sick patients at single points in time, it seemed that a reinterpretation of these findings within

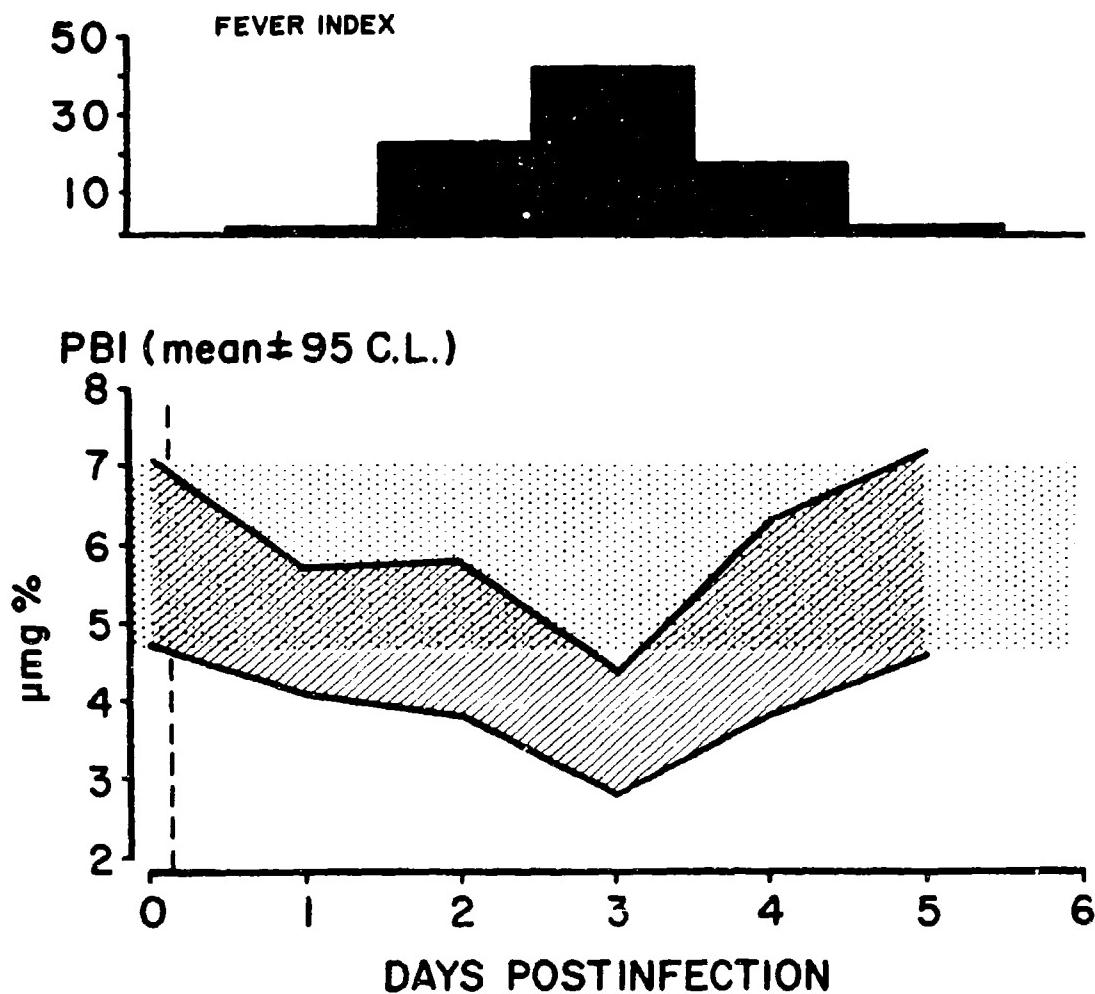


FIGURE 4. ALTERATIONS IN PBI DURING TULAREMIA
IN 8 PATIENTS.

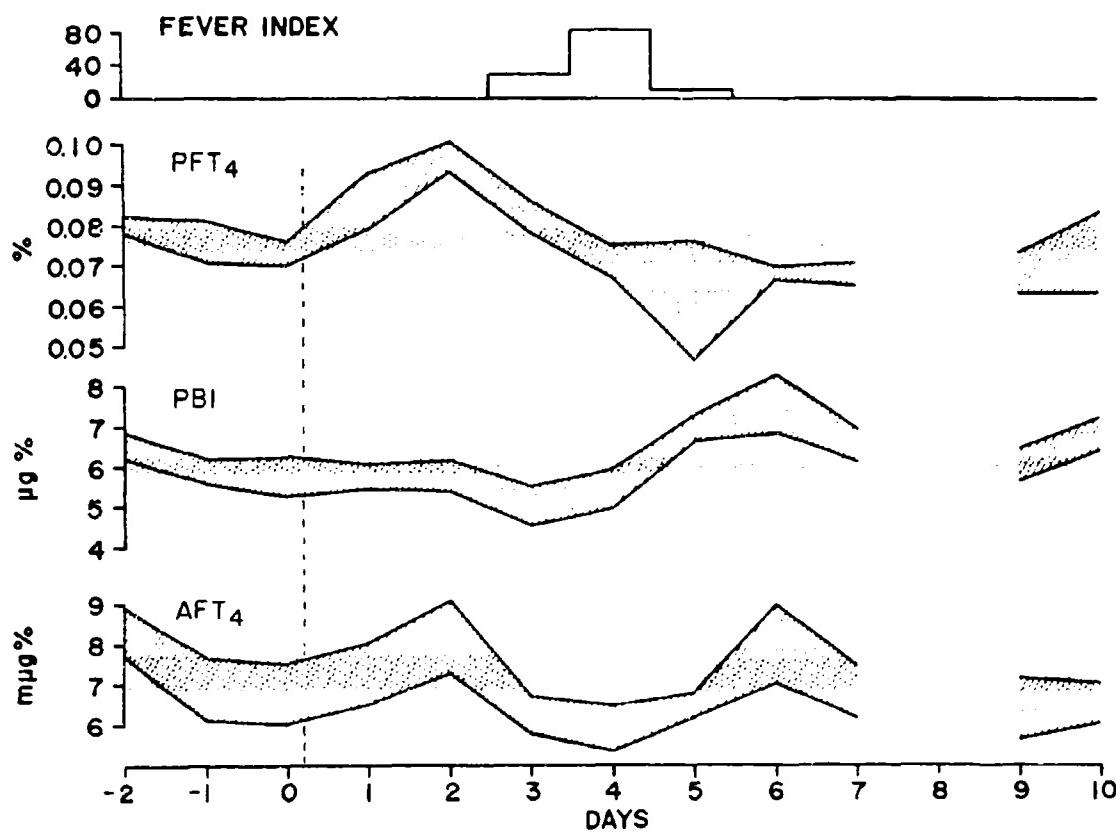


FIGURE 5. ALTERATIONS IN THYROID HORMONE PHYSIOLOGY DURING TULAREMIA IN MAN (\pm SE).

(PFT₄ = % UNBOUND THYROXINE; PBI = PROTEIN-BOUND IODINE;
AFT₄ = CONCENTRATION UNBOUND THYROXINE.)

a dynamic framework might serve to consolidate previously conflicting reports obtained from static measurements.

In Figure 6 the course of illness has been arbitrarily divided into 4 phases: subclinical infection, clinical illness (with fever), early recovery following initiation and therapy, and late recovery extending into convalescence. During subclinical infection, the percent of unbound thyroxine (PFT₄) increases and thyroxine increases and thyroxine metabolism or disposal begins to increase. By disposal we mean that quantity of hormone leaving the blood to enter peripheral tissues within a given time period. This process may result in a slight depression of the PBI. With a continued increase in PFT₄, the concentration of circulating unbound thyroxine (AFT₄) increases transiently. A fall in circulating unbound thyroxine and an accompanying fall in the PBI during fever and clinical illness may imply a further increase in thyroxine disposal. Such a fall in circulating unbound thyroxine may stimulate pituitary thyroid stimulating hormone (TSH) release with an increased thyroid gland release of thyroxine. This results in a rise in the PBI and an increase in the circulating unbound thyroxine. Following therapy and defervescence a decrease in thyroxine disposal in the face of a continuing thyroid gland output results in a sharp rise in the PBI with an increase in circulating unbound thyroxine. Stimulation to pituitary TSH-release stops, increased thyroid gland hormone output diminishes and the PBI and circulating unbound thyroxine gradually return to normal. Obviously, this concept is purely hypothetical; validation will depend upon direct measurements of thyroid gland uptake and release as well as measurements of thyroxine binding prealbumin and binding globulin and alterations in the thyroxine distribution space. In spite of these shortcomings, such a concept might help to explain why some authors^{8/} have reported a decrease in circulating unbound thyroxine and a slight fall in the PBI in sick patients, an increase in thyroxine disposal and no change in the PBI during fever; and still others^{9/} a slight increase in thyroxine disposal as well as an increase in the PBI (this last report in pneumonia patients following therapy).

Changes in thyroid hormone physiology in man during infection are definite but subtle. How might these subtle alterations in thyroid function affect the host's response to infection? Information on this point is conflicting. Severe hyperthyroidism or myxedema is generally agreed to be deleterious. A mild degree of hypothyroidism on the other hand has been associated with increased survival to a virus infection but decreased phagocytic activity and an increased susceptibility to tuberculosis. Since alterations in protein metabolism may represent a significant facet of the host response to a generalized infection it seemed possible that study of a single host protein might provide information concerning the mechanisms by which hormones such as thyroxine exert their influence during infection. Hepatic tyrosine transaminase (TT) is an enzyme with a short turnover time, easily identified and known

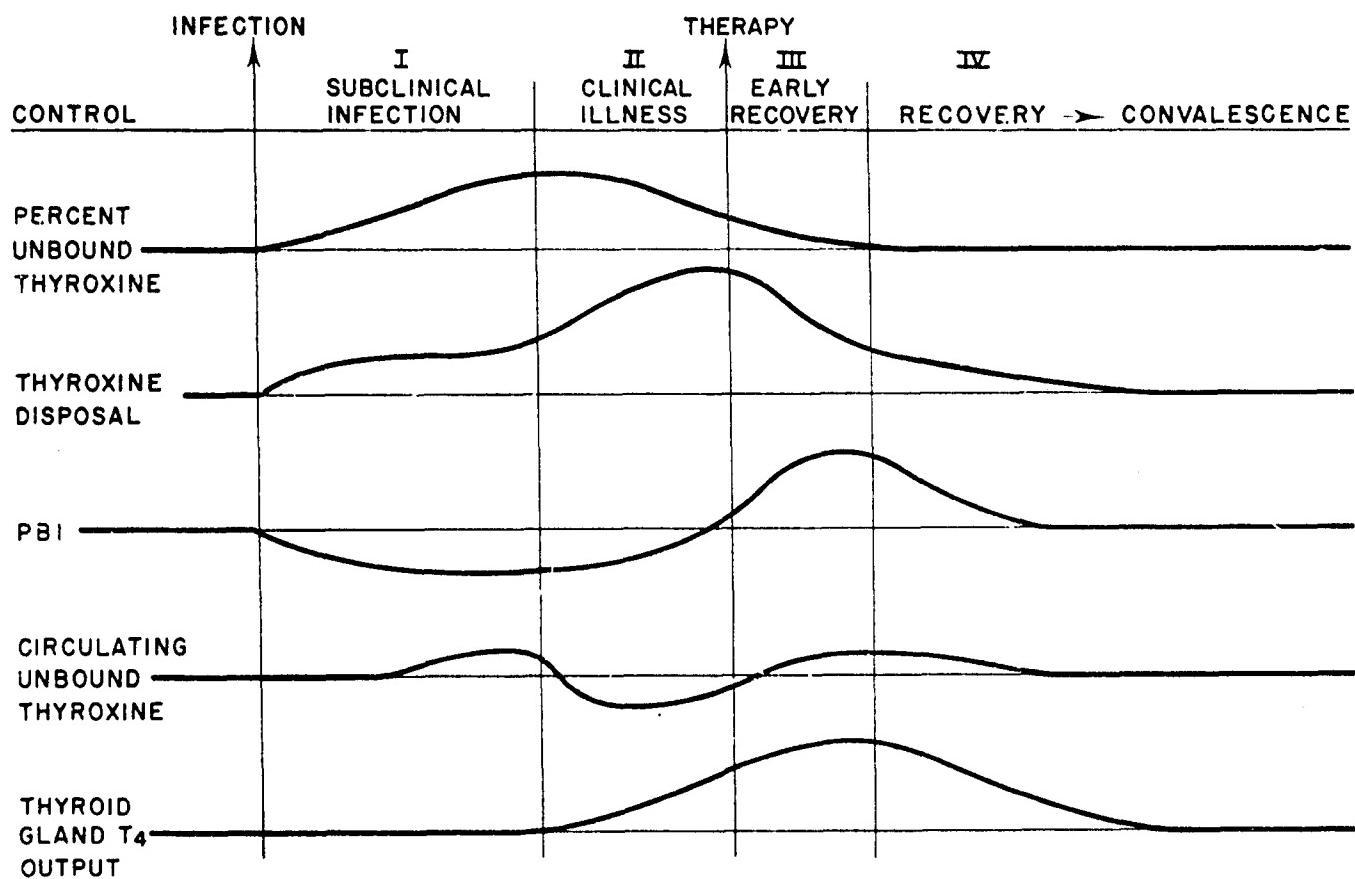


FIGURE 6. ALTERATIONS IN THYROID HORMONE PHYSIOLOGY: CONCEPT OF SEQUENTIAL CHANGES.

to be controlled in part by hormones. Recent studies in our laboratory have shown a marked increase in hepatic TT activity during pneumococcal septicemia in the rat.¹⁰ If an anabolic event such as increased de novo synthesis of this enzyme protein represents a beneficial host response, alterations in host responsiveness during hypothyroidism might be related to an alteration in protein anabolism.

To examine this possibility, thyroidectomized rats were infected and sacrificed serially every 4 hr; livers were removed and analyzed for hepatic TT activity (Figure 7). Here are shown the means \pm 1 SE of TT activity in intact rats shown in black, in infected thyroidectomized animals (cross-hatched) area, and thyroidectomized controls. Notice the 4-fold rise of hepatic TT activity in the control animals at 2200 hours. Notice also that the TT response during infection seemed to follow a pattern reminiscent of controls, but was greatly exaggerated. A diminished hepatic TT response was seen during infection in thyroidectomized animals. These findings suggest that alterations in thyroid physiology may interfere with host protein anabolism during infection. It is of interest that thyroxine does not exert a direct effect upon hepatic TT activity and that the effect seen here may be the result of modulation of an adrenocortical steroid effect. Support for the role of adreno-steroid action comes from our observations of the failure of hepatic TT activity to respond to infection in adrenalectomized or pypophysectomized rats. Thus thyroid gland alterations during infection may serve to modulate other hormonal actions.

Mechanisms underlying host response to a generalized infection are poorly defined. The actions of insulin and thyroxine may alter certain metabolic pathways. Whether, these alterations are beneficial or in the long run detrimental to the host will require further investigation.

For example, a stimulation of transaminase activity results in glutamine production, a precursor for purine biosynthesis, and urea production. An enhanced urea excretion and a concomitant decreased uric acid excretion described by Colonel Beisel¹¹ in volunteers during acute illness is strongly suggestive of a similar enhancement of transaminase activity during infection in the human. The price of an enhancement of transaminase activity may however, be a depletion of tissue amino acids.

Figure 8 shows hepatic tyrosine concentration and TT activity in rats following a low dose challenge of 50 pneumococcal organisms. During the incubation period both tyrosine and hepatic TT follow the same pattern as the controls. Notice that the hepatic tyrosine concentrations have a circadian rhythmicity that the nadir of amino acid concentrations coincides in time with maximum TT activity. This finding suggests that hepatic TT controls substrate concentration in the liver. A similar tyrosine periodicity in the small bowel mucosa suggests that hepatic TT may control the substrate concentrations in this organ as well.

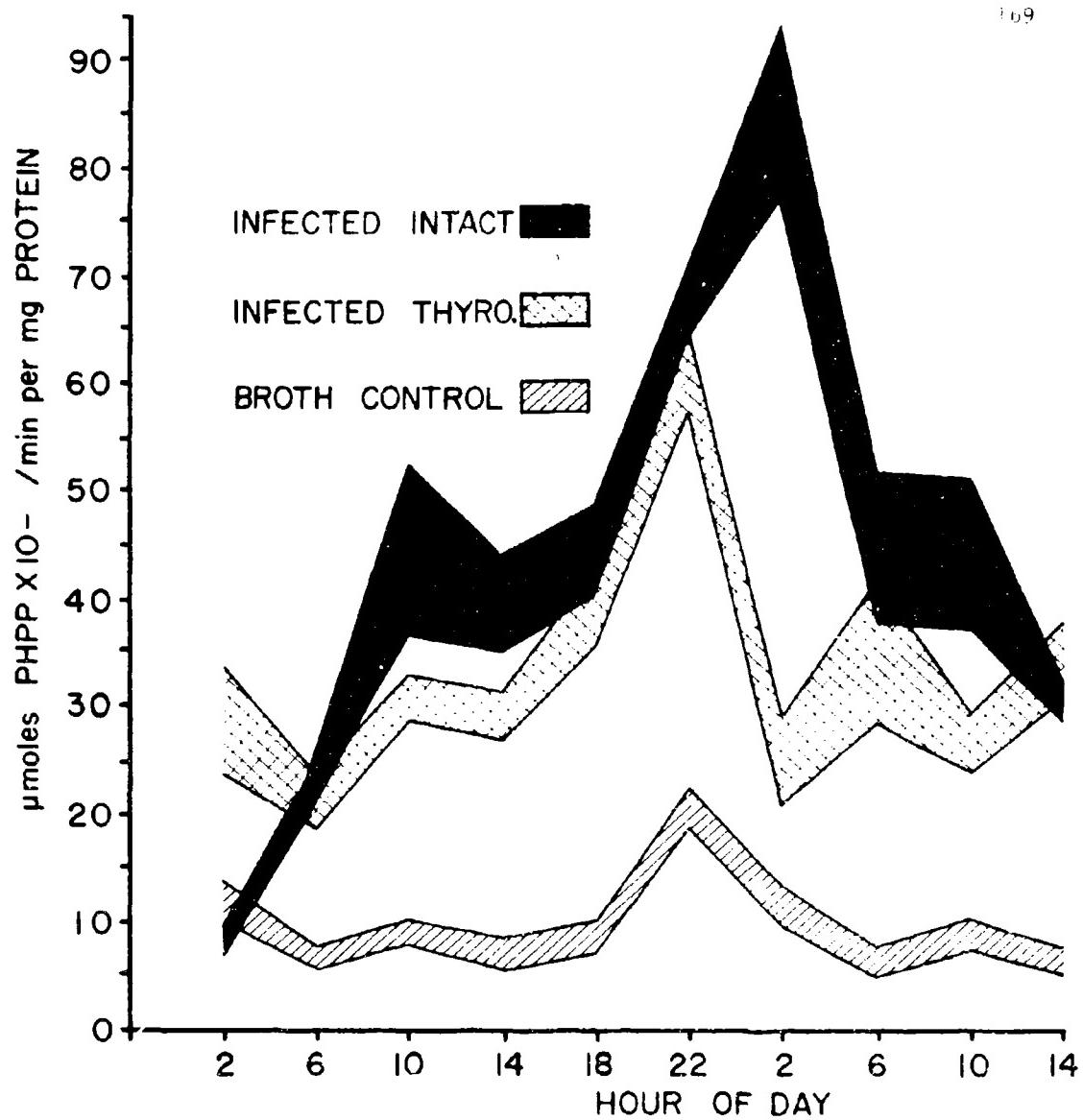


FIGURE 7. TYROSINE TRANSAMINASE ACTIVITY FOLLOWING EVENING INFECTION OF THYROIDECTOMIZED AND INTACT ANIMALS.

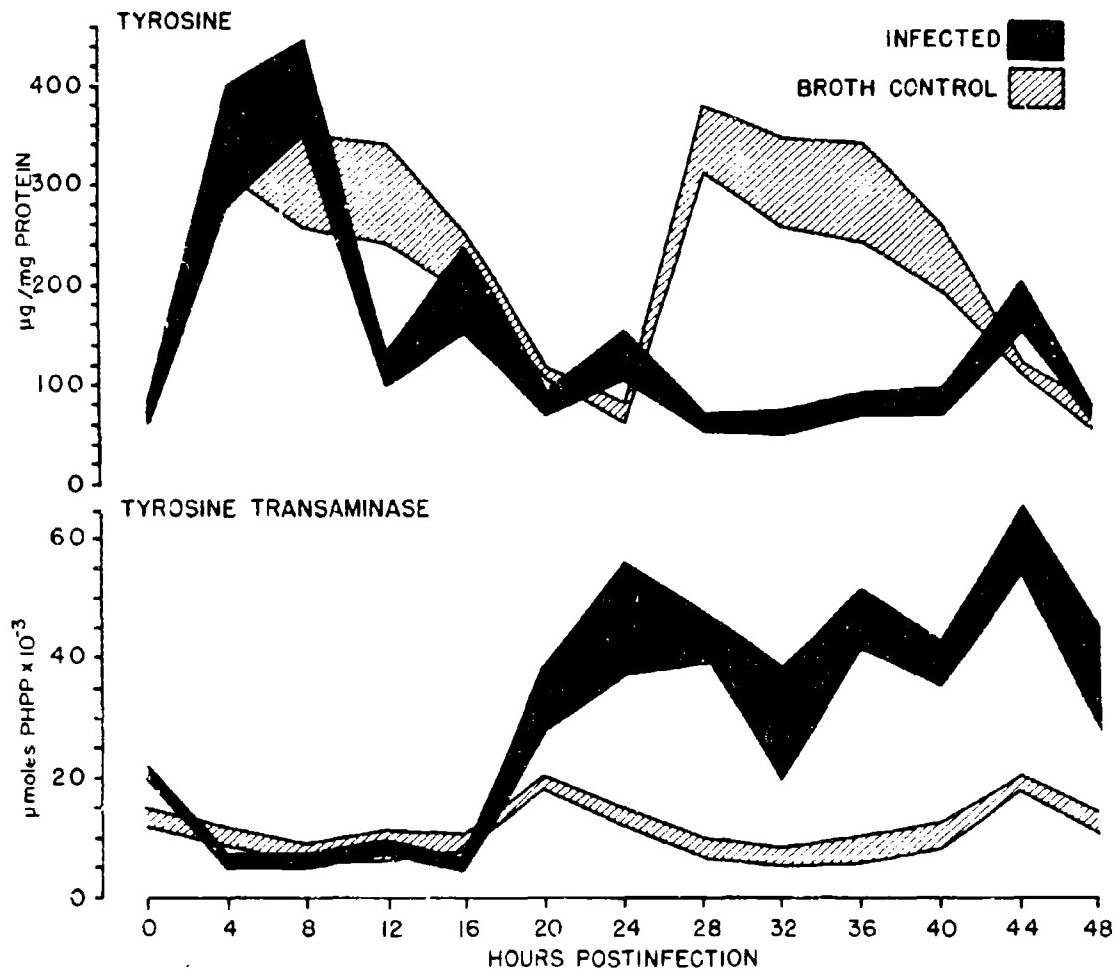


FIGURE 8. EFFECT OF LOW-DOSE INFECTION ON TYROSINE TYROSINE TRANSAMINASE ACTIVITY.

The failure of hepatic TT activity to change during the incubation period suggests that infection alone does not result in an anabolic response of this protein, and may explain the normal nitrogen balance observed in volunteers during the incubation period. During clinical illness 20 hr after infection a rise in enzyme activity is associated with a marked fall in hepatic tyrosine. Since tryptophan, methionine, histidine and alanine can also serve as substrates for this enzyme an infection-related increase in hepatic TT resulting in a similar depletion of amino acids in other tissues such as muscle may be a mechanism behind the muscle wasting seen during clinical illness.

The complex metabolic interactions comprising the alterations in the host during infection, and the multihormonal influences modifying these alterations are gradually becoming clearer. Continued correlation of clinical observations with metabolic alterations at the cellular level hopefully will provide the long sought for mechanisms underlying the observed response of the host to infection.

SUMMARY

A diabetic glucose tolerance curve seen during acute respiratory tularemia was associated with an enhanced insulin response to an IV glucose load.

Several aspects of peripheral thyroid hormone physiology were measured sequentially in subjects exposed to P. tularensis. A significant rise in the percent of unbound thyroxine was observed within 24 hr following exposure to the infecting agent. The PBI fell initially within 48 hr following exposure to the infecting microorganism. Although a depression of PBI persisted throughout the period of observation in the vaccinated group, a transient rise to concentrations significantly above controls was seen following institution of therapy. An interpretation of these observations was advanced through a hypothesis which included 4 distinct sequential changes.

These changes appeared to be nonspecific for infection per se, but provided a concept of dynamic alterations in peripheral thyroid hormone physiology that might serve to consolidate previous concepts.

Recent data obtained during pneumococcal septicemia in the rat revealed a marked enhancement of hepatic TT activity which was dependent upon an intact pituitary adrenal axis. The diminution of hepatic TT response to infection in thyroidectomized rats suggested that thyroxine modulated pituitary adrenal activity and that alterations in thyroid physiology during infection in man may modulate induction of steroid dependent proteins.

A peripheral inhibition of insulin action and an alteration in thyroid hormone physiology during acute illness may contribute to the host response to infection.

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DISCUSSION

DR. MUNRO: Your interesting and comprehensive studies on TT rhythms are particularly relevant because this field is very active just now. Did any rhythms persist in the adrenalectomized animals? I note that the fall in liver tyrosine in the control animals precedes by some hours the upswing in the enzyme activity. Does this cause a problem with interpretation of relationships between enzyme activity in the clearing of liver tyrosine?

DR. SHAMBAUGH: Thank you Dr. Munro. The answer to your first question is yes, a rhythmicity does persist in the adrenalectomized animals but it is diminished. Ordinarily, we see a 5-7-fold increase at 2200 hours above the values between 1200 to 1400 hours. In the adrenalectomized animal one sees a 2-fold increase at 2400 hours.

Your second question cannot be answered at the present time. Variability in tyrosine concentrations did not permit us to correlate the initial fall in the amino acid concentration with the upswing in enzyme activity until large differences had occurred.

DR. CLUFF: You indicated that in the convalescent period there were occasional instances where the insulin response still appeared to be exaggerated above that of normal precontrol values. As I looked at that slide, it appeared that the majority of the individuals in the group had a response which was less than that of the controls. I wonder if you have any comments to make about that.

DR. SHAMBAUGH: Initially it seemed possible that patients had been under stress at the time of precontrol study. This stressful situation involved being admitted to the ward with a "work" period prior to infection that may have prevented the full equilibration of the group. Since then, however, we have gone over our data very carefully. We have done similar insulin studies under other situations and are finding that a convalescent period decrease in insulin response may be the result of far more subtle factors. A possible relationship for such a late decrease may be the parallel changes in nitrogen metabolism in these people. This is something we have not yet been able to confirm. Although we do not have quite enough pieces to fit into a pattern, I do not think that preinfection stress alone was responsible.

DR. GRAY: Did you measure the lactate at the same time you were measuring for pyruvate?

DR. SHAMBAUGH: No, we did not.

DR. CLUFF: Would you care to speculate about what you might find with the lactate pyruvate difference?

DR. GRAY: In a variety of stress situations, particularly in ones in which there may be a shift toward anaerobic metabolism, plasma lactate goes up even further than does the pyruvate, so one might expect a lactate increase greater than that of pyruvate. On the other hand, there have been reports that the lactate dehydrogenase activity goes up in infection. If so, one might speculate that the increased pyruvate was at the expense of the lactate. So it would be interesting to see which way the lactate did go.

ADRENOCORTICAL RESPONSE AND INFECTIOUS DISEASE

Colonel William R. Beisel, MC*

In his recent book, "Adrenal Steroids and Disease," Dr. Cuthbert Cope commented at some length on the interrelationships between adrenal function, stress, inflammation, and immunity. One particular passage is worthy of direct quotation: "It is well recognized that the phenomena of inflammation as well as those of resistance to infection are highly complex and closely integrated. It is not surprising, therefore, that the action of the corticosteroids cannot be defined as affecting any particular stage or any one enzyme system. On the contrary, the corticosteroids seem to influence in an insidious but nevertheless detectable manner, most of the different aspects of disease resistance which have been studied. No simplified concept of this action can as yet be achieved; possibly it never will be."¹ To this rather pessimistic appraisal is added the comment that the elucidation of these obscure but clearly important mechanisms still remains a major challenge for investigators in the future.

Since this challenge has stimulated the work of many individuals at this meeting, it seems germane to review, in brief, the current status of our understanding of the several interrelationships between infectious illness and the adrenal cortex.

The impetus given to studies of adrenal physiology by Selye,^{2,3} followed by extensive investigations (conducted during the early 1950's) permitted the development of many basic concepts which remain as fundamental components of our current thinking and knowledge. The availability of new data, however, permits a reappraisal of several areas which I will attempt to review in the manner of a progress report. First, consider the impact of acute infection on the adrenal cortex. It has long been recognized that the adrenal cortical cells become unusually active during acute infection as evidenced by alterations in their histologic appearance and by the depletion of the adrenocortical lipids and ascorbic acid; on occasion, adrenocortical cells may undergo hemorrhagic necrosis. While many concepts have been advanced concerning pathogenic mechanisms on such an acute adrenal destruction, the recent work of Levin and Cluff⁴ has for the first time provided a reproducible model system by which to study this phenomenon in detail; these workers have shown that the cortex is subject to necrosis following bacterial endotoxin administration, but only if it is functioning at a stimulated rate at the time of this administration. Apparently, the nature of the initial stimulus is not important, but rather, the presence of a prestimulated cortex.

* U. S. Army Medical Unit.

Next, consider the nature of the adrenocortical response to infection. Despite the evidence of Conn and co-workers^{5/} that different forms of stress produce markedly different patterns of adrenal response, and the fact that very little has been published until quite recently concerning adrenal function during infection, most comments or statements of "common knowledge" give the impression that acute infectious stress produces a maximal stimulus to adrenal activity. A corollary to this has been the concept that adrenal overactivity contributes, in large part, to the catabolic aspects of infection. Our own work, as presented in an earlier meeting of this Commission, tends to refute both of these concepts.

As determined in prospective studies involving the exposure of volunteers to Pasteurella tularensis,^{6/} Coxiella burnetii,^{7/} or sandfly fever virus,^{7/} acute infection did produce an adrenocortical response of limited magnitude and brief duration. In these infections, shown in Figure 1 for tularemia, a rather stereotyped response occurred exhibiting the following characteristics: (1) Increased glucocorticoid hormone output began coincident with or shortly before the onset of fever and symptoms. The increase rarely exceeded baseline output, and reverted abruptly to normal with the onset of clinical recovery. (2) Plasma corticosteroids lost their afternoon diurnal fall and remained at or slightly above the usual early morning concentrations. There was no change in their binding by plasma proteins. (3) Ketosteroid and pregnanetriol excretion changes were less prominent than those of the glucocorticoids; and (4) Increased excretion of aldosterone lagged behind the glucocorticoid response in its timing, and coincided with the well recognized fall in urinary Na and Cl during infection. Mild infectious illness may fail to produce a detectable adrenal response.

While all of our subjects exhibited disease that was short in duration and generally only prodromal in severity, other evidence indicates that severe or protracted acute infections, or chronic infection, associated with only a comparable degree of increase or an actual depression of adrenal function. Repeated observations by investigators^{8/} from Walter Reed Army Institute of Research recently showed that there was often a depressed, or at best, only a normal output of adrenal hormones during the course of severe falciparum malaria in previously healthy American soldiers. Even in septicemic shock, the terminal marked elevations of plasma corticoids may be attributed to a failure of the liver to remove and metabolize the adrenal steroids, rather than to markedly high adrenal secretion rates.

These descriptive remarks are compatible with the meager amounts of hard data uncovered by an extensive and prolonged literature research. In considering mechanisms behind these changes, little is known about the exact status of the anterior pituitary during infectious illness; available reports of ACTH or methopyrapone infusions during severe or chronic infections have not provided consistent patterns of response through which to implicate either an impairment of pituitary or adrenal function as a principal defect.

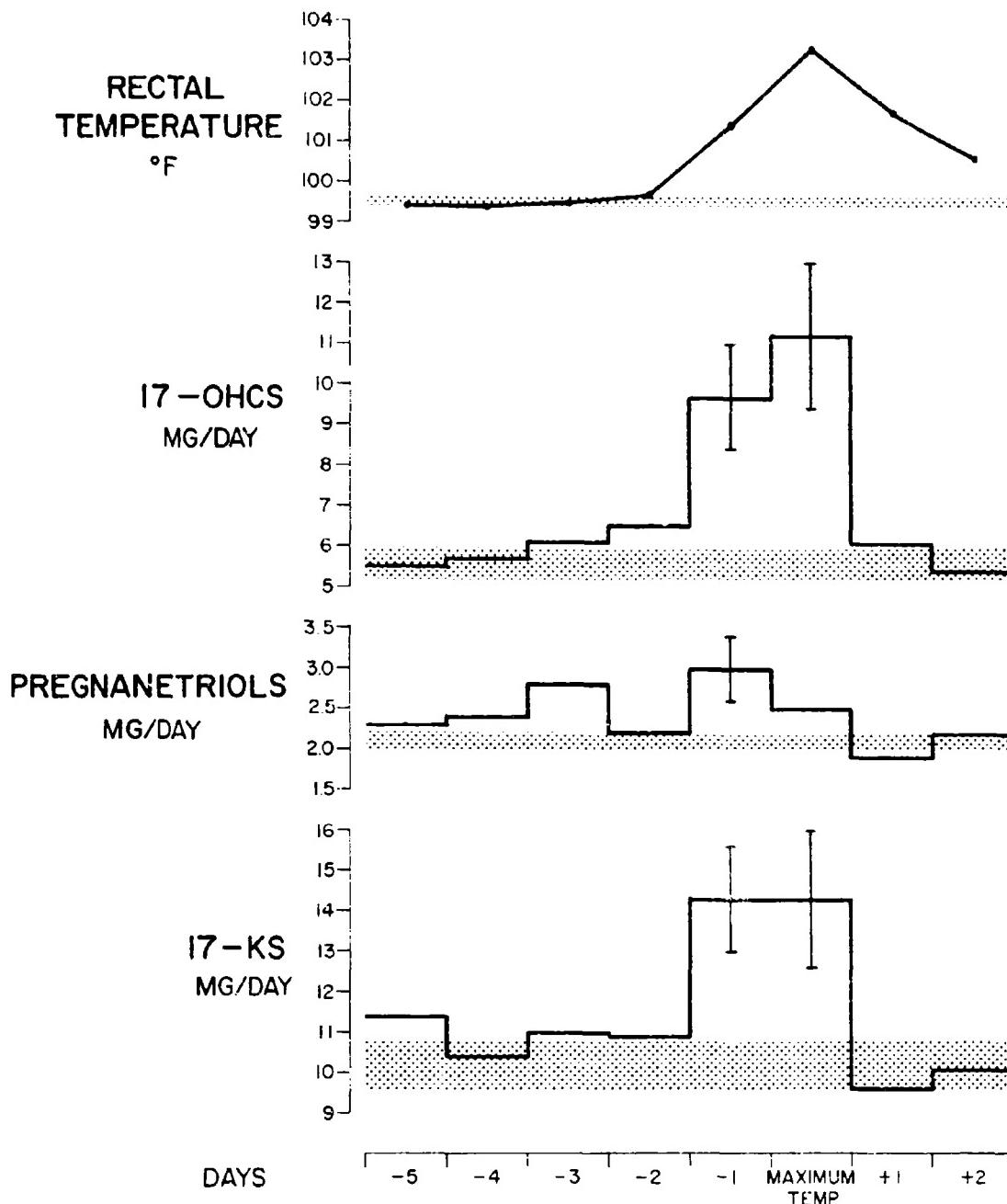


FIGURE 1. URINARY CORTICOSTEROIDS IN 12 SUBJECTS WITH ACUTE TULAREMIA.

Now let us consider the mechanisms through which adrenal functional changes may support or impair resistance of the host. As noted above, there are indications of an adrenal effect on most of the various inter-related aspects of inflammation, phagocytosis by mobile cells as well as the reticuloendothelial system, interferon production, and the antibody responses. Many of these effects, including adrenocortical actions on lysosomal membranes, have been demonstrated only after the use of high, nonphysiologic doses or concentrations of hormone. By their very nature, well designed investigative models generally require rigid controls or in vitro conditions to permit exact definition of a specific host-micro-organism interaction. The impact of glucocorticoid hormones under such artificial circumstances often cannot readily be interpreted in terms of the intact host. On the other hand, studies which compare physiological or biochemical changes in the infected host with simultaneous measurements of adrenocortical hormones cannot in themselves establish clear-cut cause-effect relationships, however close the temporal correlations.

Concerning the influence of adrenal hormones on host resistance, I would suggest that for many years investigation regarding the cellular actions of glucocorticoids has addressed itself to the wrong question. Demonstrations by Kass and Finland^{9/} over a decade ago indicated clearly that host resistance was optimal under eucorticoid conditions, and that either a deficient or an excessive concentration of glucocorticoids was harmful to the host. Despite several early studies that showed a beneficial effect of very small doses of cortisone in animals with experimental bacterial infections, investigators consistently became fascinated by dramatic changes in a variety of systems which accompanied the administration of large, pharmacologic doses of a potent glucocorticoid. Such experimental designs resulted in clear-cut changes and publishable data, but all too seldom permitted interpretation in terms of responses in a normal host. To me, the fundamental, long neglected question should be, "How does the normal endogenous adrenal response to infection provide benefit for the host?"

To be sure, we do need information concerning the pharmacologic effects of high-dose steroid therapy, especially when these steroids are employed specifically for their immunosuppressive action as in organ transplantation. My complaint here, is that the adrenal role has been neglected as it pertains to normal circumstances within the physiologic range. It seemed most likely that the answer to the latter question was related to alterations in protein metabolism.

Wasting of body nitrogen constitutes a major metabolic response of the host during infectious illness. Based on classic concepts it was logical to assume that the infection-related increase of glucocorticoid activity would contribute importantly to protein catabolism and increased nitrogen loss. However, such a single direct interaction is conceptually inadequate to account for all of the known changes in nitrogen metabolism during infection, and is probably only small in overall importance. The

magnitude and duration of glucocorticoid excess observed repeatedly during infection seemed far too small to explain catabolic losses measured during acute brief illnesses. When the pattern of glucocorticoid increase during acute tularemia was reproduced quantitatively by the oral administration of cortisol to healthy noninfected normal subjects over a period of several days, no loss of body nitrogen became evident (Figure 2). I believe that Dr. Vernon Young, our next speaker, will present evidence from his studies of muscle cell ribosomes giving excellent new data on this point.

Other evidence presented at this meeting suggests that endogenous glucocorticoids may be beneficial to the host by stimulating increased protein anabolism, especially within the liver.

Data of Dr. Rapoport (Section I) showed that the usual infection-related increase in hepatic tryptophan pyrrolase (TP) activity failed to occur in adrenalectomized mice even if they were given replacement corticoid therapy.

An analogous situation was found by Dr. Shambaugh^{10/} for another hepatic enzyme known to be induced rapidly by glucocorticoids. This enzyme, tyrosine transaminase (TT), also was induced rapidly by pneumococcal infection of rats as shown in black in Figure 3 which represents the mean \pm 1 SE of groups sacrificed serially. This rise in enzyme activity followed at an appropriate interval the rise in plasma corticosterone due to increased adrenal secretion. In low dose inoculations, the rise in TT activity did not begin until after the onset of bacteremia, fever, and an adrenal response. Neither adrenalectomized nor hypophysectomized animals showed such an enzyme response; rather, their values remained at all times after infection below those of the control groups. Changes in hepatic TT, like those of hepatic TP, thus appeared to represent responses secondary to an increase in adrenal glucocorticoids. One might wonder about a similar mechanism for several of the very early enzyme increases reported by Dr. John Woodward. I wonder if this early mechanism might be different from the late rises of cellular enzyme that might be ascribed to cellular destruction.

One cannot assume from these data that all enzymes known to be induced by cortisol will respond in a similar way. Indeed, the opposite is true.

Changes in leukocyte activity of alkaline phosphatase during typical tularemia in volunteers are shown in Figure 4. Note that the pattern of response was much slower than that of the glucocorticoid response. In a control study, a single day of environmentally induced fever in volunteers produced an adrenal response equal to that observed in tularemia along with prominent leukocytosis but it failed to result in altered white blood cell alkaline phosphatase.^{11/} Further, increases of alkaline phosphatase in the intestinal mucosa of mice infected with pneumococci occurred with equal prominence in either intact or adrenalectomized mice.

Thus, our studies of enzyme response to infection suggest that adrenal activity is probably only one of a number of regulatory factors.

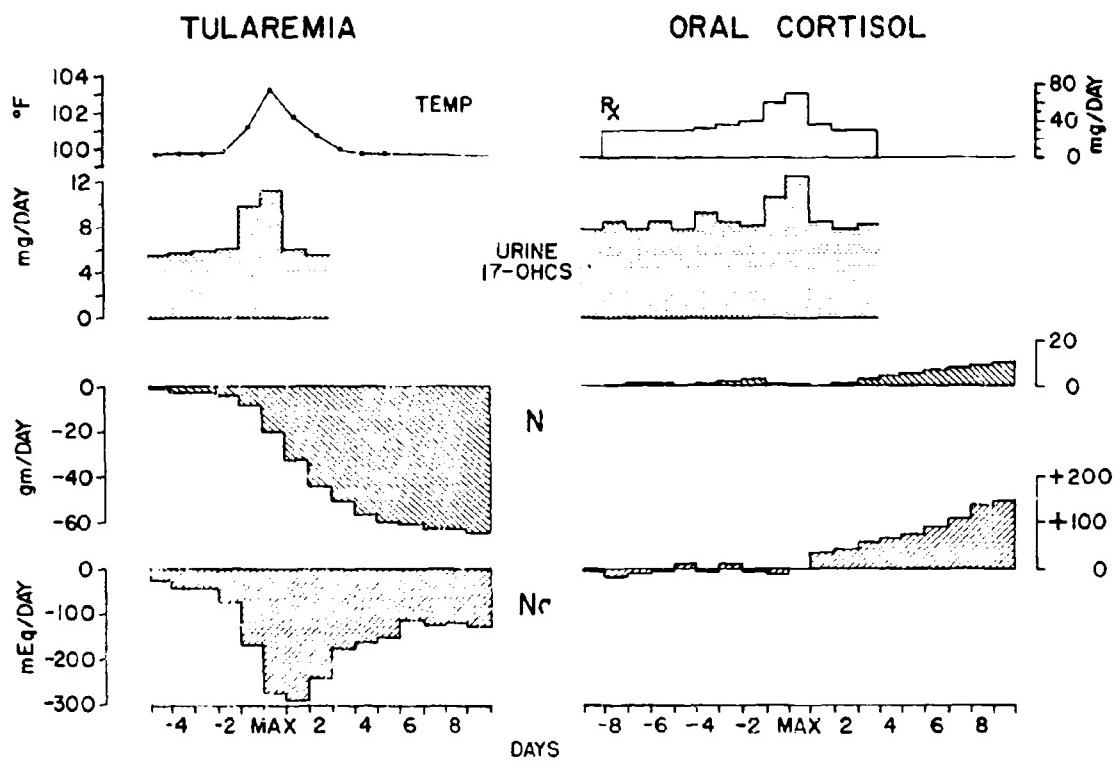


FIGURE 2. EFFECT OF ORAL CORTISOL DUPLICATING URINE 17-OHCS EXCRETION OF TULAREMIA OF NITROGEN AND SODIUM BALANCES.

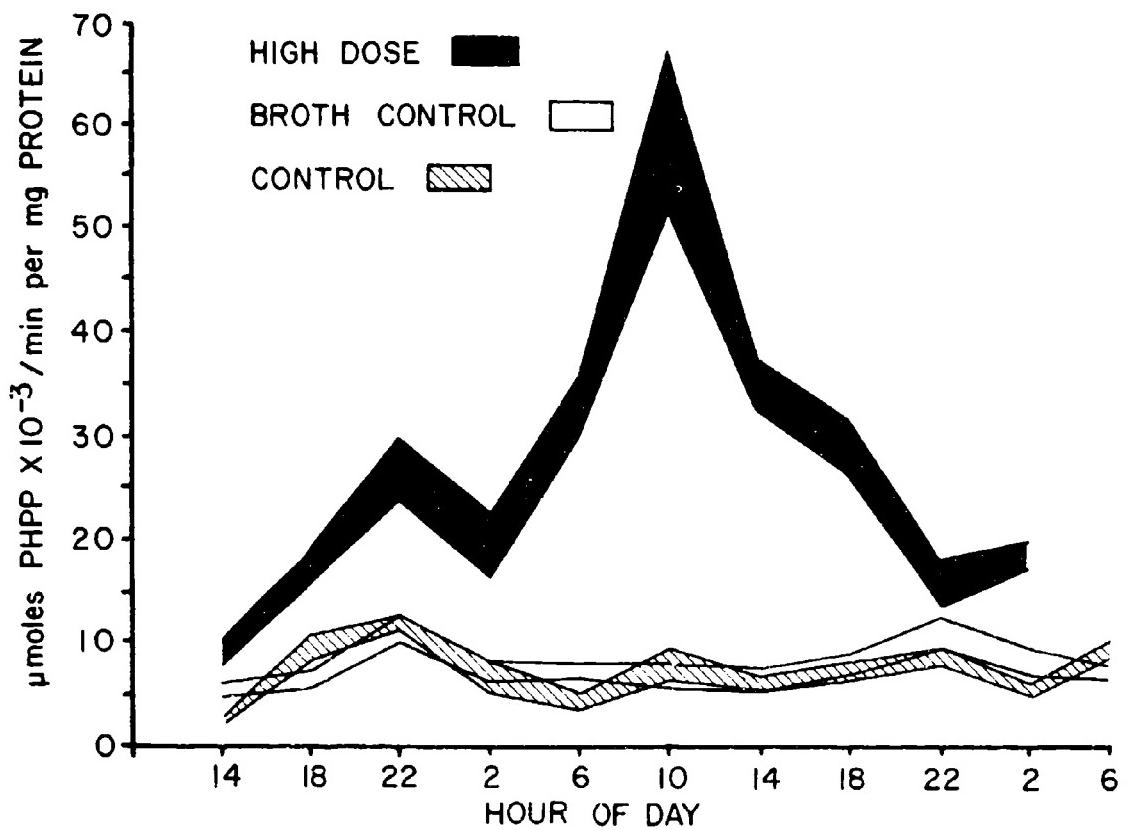


FIGURE 3. TYROSINE TRANSAMINASE ACTIVITY FOLLOWING MORNING INFECTION WITH *D. PNEUMONIAE*.

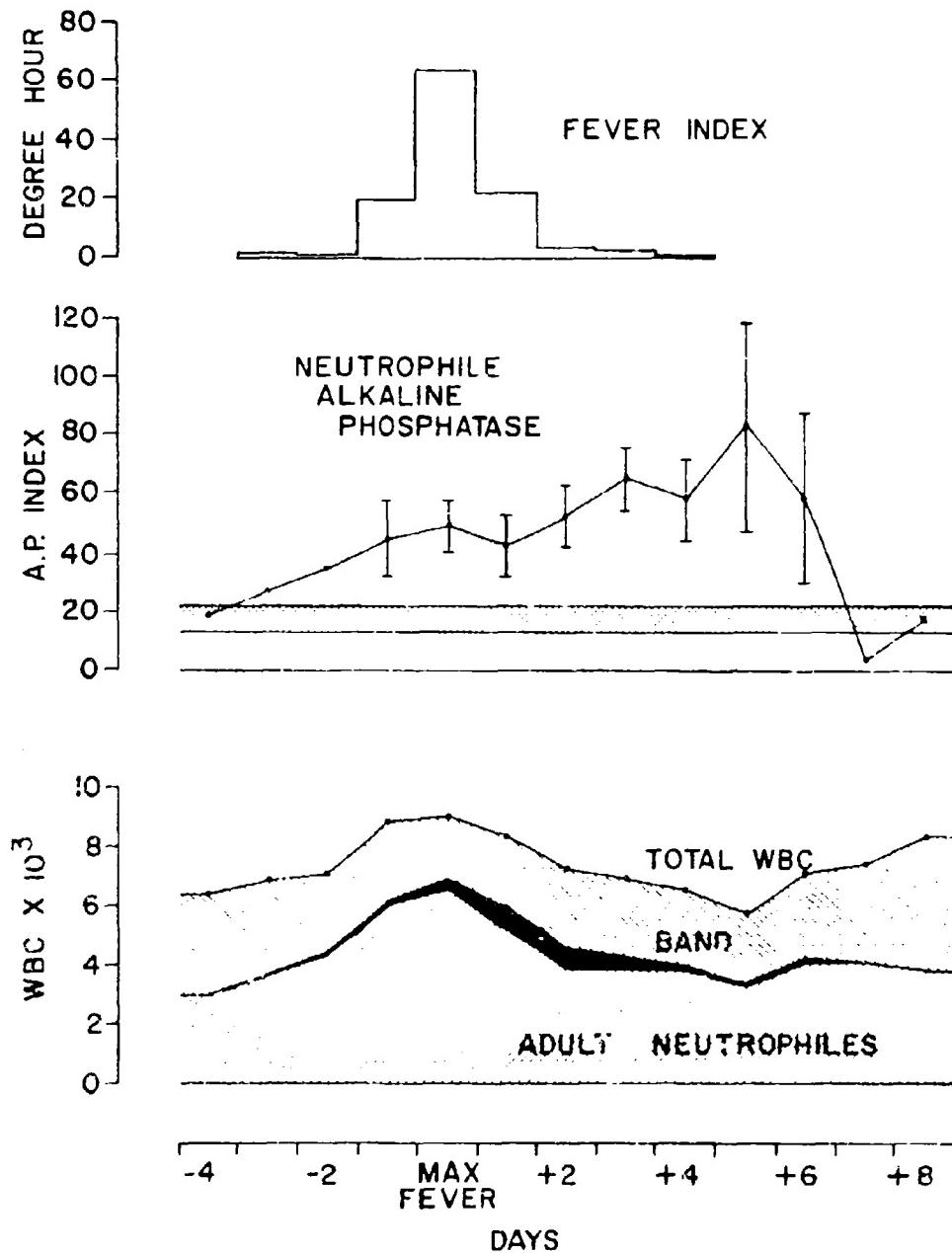


FIGURE 4. NEUTROPHILE ALKALINE PHOSPHATASE RESPONSE
IN TYPICAL ACUTE TULAREMIA.

SUMMARY

A variety of new data has recently become available, making it possible to review and reevaluate long-held concepts concerning the interrelationships between adrenal function and infectious illness. Evidence has been presented suggesting that the beneficial role of the normal adrenal gland involves its ability to induce new proteins within the host. At the present time such a concept is valuable as a lead for future work; however, it is too early to claim that the increase of any specific protein induced by corticosteroids has a clear-cut effect on host resistance.

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DISCUSSION

DR. T. E. WOODWARD: My question pertains to the matter of necrosis in the adrenal gland and the hyperreacting gland. In Melby's article concerning plasma glucocorticoid levels in patients who were stressed with severe Gram-negative infections, was it not shown that persons who died had the highest levels? And that they did respond to exogenously given ACTH?

DR. BEISEL: Yes, that is correct. The patients who died did have very high concentrations. Further, infused radioactive cortisol showed a much delayed clearance from the blood stream in these individuals. This fact lead to the interpretation concerning a failure of hepatic conjugation of corticosteroids during Gram-negative shock. Melby and Spink also showed that by ACTH infusions they could stimulate the adrenal cortex of such a sick individual to an even greater output. This gave an indication that the adrenal cortex was not producing maximally, despite the high corticoid concentrations in the blood of dying patients.

DR. T. E. WOODWARD: Did those adrenal glands show necrosis at autopsy? I don't remember.

DR. BEISEL: To my knowledge, they did not.

DR. MUNRO: With regard to the adrenal cortical activity in infection, it would appear that your results now mimic responses during reaction to trauma, i.e., that the disturbance in nitrogen metabolism is prolonged whereas the effects on blood levels and excretion of cortical steroids is relatively brief. In that case, animal experiments carried out by Engle many years ago and repeated by at least one other group demonstrated that the adrenal cortex played a permissive role. This is, excretion of adrenal corticoids was necessary, but not necessarily the effective factor in causing nitrogen wasting. Now is there any comparable evidence? Or indeed, is there any reason to continue to hold the view that Engle put forward concerning the permissive role of the adrenal cortex in the case of febrile subjects? Or animals?

DR. BEISEL: This is a very searching question and we have given it much thought. We attempted to devise a model to document this, i.e., the TP response in mice, as reported by Dr. Rapoport (Section I). Physiologic replacement of corticosterone was maintained in adrenalectomized animals to determine if the infection-related, and apparently steroid-related, rise in TP would occur. It did not. This implied to us that there was no permissive role for at least this anabolic event. We have not looked at the catabolic phase of infection in a similar manner, so that remains an

unanswered question. Certainly there are many similarities between the adrenal response to trauma, operation, burns, etc. and the response to infection in our subjects.

DR. T. E. WOODWARD: If I'm not mistaken, there have been recent attempts to study young children with meningococcemia with correlative analyses of the corticosteroid levels in the blood and comparison with the pathological material that might be available at autopsy. If I'm not mistaken, the corticosteroid level of the blood is lower than normal in children who die with adrenal hemorrhage. But in those who die without any evidence of adrenal hemorrhage, cortical steroid levels are high. How would one fit such evidence into the interpretation that you're giving, that there is a decreased removal of corticosteroid by the liver? Even if there is a terminal adrenal hemorrhage, an excessive steroid level should have been reached prior to the hemorrhagic and necrotic reaction of the adrenal gland. This combination should have resulted in perpetuation of a high level corticosteroid.

DR. BEISEL: I believe that the time relationships here are most important. Deaths due to acute meningococci often occur within less than a 24 hr period of initial symptoms. A glucocorticoid buildup before the adrenal destruction may not reach the exceedingly high levels that develop in patients who have been in Gram-negative septicemia shock for a period of several days. Most published work is based on 1 or 2 well studied cases; certainly more information is needed in this area.

DR. CLUFF: You showed the changes in AP activity of granulocytes in comparison to the total granulocyte count. At the time AP was rising progressively in the white cells there was no appreciable change in the total neutrophil count. If the cortical steroid is responsible for the increased AP activity of the granulocytes, can this be induced in vitro?

DR. BEISEL: To my knowledge AP cannot be induced in vitro in the white cells. A clinical, white cell AP response to glucocorticoids required large doses of the hormone maintained for a period of several days. Dr. Valentine did most of this work about 8 or 9 years ago and in each instance he used prolonged doses of the hormones. This particular enzyme has been shown, however, to change in other tissues of the body as a result of the adrenal hormones.

DR. CLUFF: Then it isn't known conclusively that the physiological rises in corticosteroid levels associated with infection are indeed responsible for the increased AP activity of the granulocytes.

DR. BEISEL: In my estimation, such an AP rise seen late in infection did not seem to be related to adrenal steroids; the mechanism behind the AP response in leukocytes remains unknown.

MUSCLE PROTEIN METABOLISM

Vernon R. Young, Ph.D.*

It has been well established that generalized infection leads to an increased rate of urinary nitrogen excretion and a depletion of body protein.^{1,2,3/} Furthermore, the extent of N loss appears to depend upon prior protein intake.^{3,4/} While the source of the loss following the stress of infection has not been studied in detail, investigations in experimental animals^{4,5/} and man^{3/} suggest that muscle supplies a major proportion of the N.

The mechanisms which lead to the changes in muscle protein metabolism have not been investigated. Therefore, studies have been undertaken to examine the changes which occur in muscle protein metabolism following experimental infection with Salmonella typhimurium in the rat. A number of reaction sequences are associated with protein synthesis, and it may be that one or more of these are effected by a generalized infection. In the present studies, particular emphasis has been given to the ribosome in view of the central role played by this subcellular particle in the protein synthesis of mammalian tissues.

A number of parameters of muscle protein synthesis were studied in the present experiments. The capacity for incorporation of C¹⁴-labeled amino acid into nascent peptides by ribosomes both in vivo and in vitro has been investigated. The sedimentation of muscle ribosomes in sucrose gradients was also studied as a qualitative reflection of the changes in muscle protein metabolism.

Male, weanling rats (Sprague-Dawley strain) were used. They were fed an adequate, 18% casein diet for at least 3 days before use. Our initial experiments included a study of the effects of diet, glucocorticoids and insulin on the size distribution of ribosomes present in a postmitochondrial supernatant fluid prepared from rat skeletal muscle.

The first study was conducted with rats given the 18% casein diet throughout the experimental period. The second was conducted with rats given a 5% casein diet for 9 days prior to infection and their pair-fed mates given an 18% casein diet. S. typhimurium organisms came from a culture initially isolated by Professor P. M. Newberne from our rat colony. Each rat was given by intraperitoneal (IP) injection of an aliquot of an 18h-trypticase-soy-yeast broth culture (0.1 ml/100 gm body wt). The design of the infection experiments is shown in Table I, and details of the other experiments are given in the results.

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TABLE I. DESIGN OF STUDIES ON SALMONELLA TYPHIMURIUM INFECTION IN RATS

EXPERIMENT NO.	GROUP	NO. PER GROUP	DIETARY CASEIN (%)	STATUS OF INFECTION	FOOD INTAKE	PURPOSE
1	18-C-1	20 ^{a/}	18	Absent	pair-fed to 18-I-1	Biochem.
	18-I-1	20	18	Present	ad lib.	Biochem.
	18-I-1b	20	18	Present	ad lib.	Observation
2	5-I-2	80 ^{b/}	5	Present	ad lib.	Biochem.
	18-I-2	80 ^{b/}	18	Present	pair-fed to 5-I-2	Biochem.

a. 4 rats used as initial controls.

b. 10 rats used as initial controls.

The Sedimentation of Muscle Ribosomes in Sucrose Gradients.--Rats were decapitated with a guillotine, the muscle from the rear legs was removed. After the muscle had been cleaned quickly of adhering fat and connective tissue, it was immersed in chilled Medium A of the following composition: 0.25M KCl, 0.01M MgCl₂, and 0.01M tris-HCl buffer, pH 7.6. All subsequent operations were performed in a cold room at 4 C. The muscle from each rat was blotted and pooled-samples were prepared from 3-5 rats. Usually, a total of 4-6 gm of muscle was taken and transferred to a chilled 50-ml beaker containing 5 ml of Medium A. The sample was minced finely with a pair of scissors and homogenized in 2 vol of Medium A for 20 sec with the aid of a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). The homogenate was centrifuged at 15,000 rpm in a Rotor 30 of a Spinco Model L ultracentrifuge for 15 min to sediment the cell debris, mitochondria and nuclei. The supernatant fluid was filtered through 2 layers of nylon cloth (Nitex 230T, Lambert Silk Screen Co., Boston, Mass.), moistened with Medium A. The filtrate was then treated with 10% (w/v) Lubrol WX dissolved in Medium A and a 10% solution of sodium deoxycholate (in distilled water) to achieve a final concentration of 0.5% Lubrol WX and 1% deoxycholate.

The resulting fluid, usually 0.7-0.8 ml (equivalent to approximately 0.4 gm initial tissue), was then layered on a linear 15-40% sucrose gradient, 28 ml prepared in Medium A according to the method of Britten and Roberts.^{6/} The gradient was centrifuged in the SW 25.1 Rotor for 2 hr at 25,000 rpm. Absorbancy profiles were measured at 260 m μ with the aid

of a flow-cell and a Gilford Model 2000 Absorbance Recorder (Gilford Instrument Laboratories, Oberlin, Ohio). The recorder was first set at 0 against the 40% sucrose medium. The gradients were removed at the rate of 3.4 ml/min from the bottom of the tube.

Preparation of Polyribosomes.--Polyribosomes were prepared for in vitro experiments by the addition of 3 vol of buffer, Medium B (0.01M-MgCl₂, 0.01M-tris-HCl, pH 7.6) to 1 vol of the detergent-treated-postmitochondrial supernatant fluid as prepared above. The mixture was kept at 4 C for 15 min and then centrifuged for 10 min at 10,000 rpm in the Rotor 30. The white pellet was carefully resuspended in Medium A with 4 strokes of a loose pestle in an all-glass Dounce homogenizer. The suspension was centrifuged for 60 min in a Rotor 40 at 40,000 rpm. The pellet was again resuspended in Medium A; the suspension was used for studies of amino acid incorporation in the cell-free system or for evaluation of in vivo studies. The yield of polyribosomes was approximately 0.3-0.4 mg of RNA/gm muscle tissue.

Radioactive labeling of Nascent Polypeptide Chains In Vivo.--Intact rats were injected intramuscularly (IM) in each hind leg with 10 μ c of a C¹⁴-labeled amino acid mixture. They were sacrificed 15 or 45 min later; the muscle was prepared for sucrose gradient analysis as already described. Fractions of the gradients were collected in tubes kept at 4 C. For measurement of radioactivity, 0.25 mg of bovine serum albumin was added to each tube, the protein precipitated by addition of ice-cold 5% trichloroacetic acid (TCA). The tubes were kept overnight at 4 C. The precipitates were collected on 0.45-h Millipore filters and washed twice with cold 5% TCA. The filters were dried and counted on stainless steel planchets in a Nuclear-Chicago gas-flow counter.

Cell-Free Amino Acid Incorporation.--The system used for these studies was as follows: 5 μ moles tris-HCl, pH 7.6, 10 μ moles MgCl₂, 70 μ moles KCl, 2 μ moles ATP, 1 μ mole GTP, 15 μ moles creatine phosphate, 20 μ g creatine phosphokinase, 1.0 mg of pH-5 enzyme fraction, and 10 μ moles of a complete amino acid mixture containing 1 μ c of a uniformly C¹⁴-labeled amino acid mixture. The pH-5 fraction was prepared from liver and muscle tissue according to the method of Keller and Zamecnik.⁷ A final volume of 1 ml was used for the in vitro assay system. The polyribosome fraction was added at 0 time and tubes incubated in air at 37 C in a Dubnoff metabolic shaker for various time intervals. At the end of the incubation periods, the proteins were precipitated by adding cold 5% TCA; the tubes were kept at 4 C for 30 min. The tubes were heated at 56 C for 30 min, and the precipitate was washed twice with the cold TCA and once with absolute ethanol. The washed precipitate was then dissolved in 1 ml of hydroxyhyamine; 10 ml of 0.6% 2,5-diphenyloxazole in toluene was added; the radioactivity was assayed in a liquid scintillation counter. Correction was made for 0-time controls. The assay was linear with respect to ribosomes up to 0.2 mg ribosomal RNA.

Leucine-C¹⁴ Uptake by Rat Skeletal Muscle Ribosomes In Vivo.--Rats were given C¹⁴-leucine (10 μ c/100 gm body wt) by IP injection and sacrificed 3 hr later, a time at which peak specific activity is associated with the ribosome fraction. Ribosomes were prepared as above and assayed with Bray's solution⁸ in a liquid scintillation counter.

Uptake of Leucine-C¹⁴ into Mixed Liver Protein.--Liver was homogenized with 10 ml of cold distilled water and centrifuged in a Rotor 30 for 15 min at 15,000 rpm. The supernatant protein was precipitated, washed and prepared for counting as before.

At the onset of our studies, it was necessary to attempt a method which would allow close examination of the distribution of ribosome aggregates present in the muscle cell. Use of buffer systems normally applied to studies of liver protein metabolism was found to result in a low yield of muscle polyribosomes. However, the application of the buffer used by Heywood et al.⁹ allowed the preparation of reproducible profiles of ribosomes from rat skeletal muscle. In accordance with their findings in chick embryo tissue, the KCl molarity of the initial homogenizing medium had a marked influence on the yield of muscle polyribosomes. These results are illustrated in Figure 1.

The distribution of radioactivity in the profile was then examined 15 and 45 min after IM injection of a C¹⁴-labeled amino mixture in weanling rats. These results (Figure 2) demonstrate that, after a short pulse, activity is associated with the heavy polyribosome region of the gradient and after a longer period of incubation *in vivo*, activity then becomes associated with the lighter regions of the gradient. These results suggested that the method of preparation of the muscle ribosomes for sedimentation analysis did not result in a marked breakdown of polyribosomes and that the profiles obtained would reflect the extent of ribosomal aggregation in the muscle cell following various experimental treatments.

Initially we conducted a number of studies on the effects of diet and hormones on the distribution of ribosomes in skeletal muscle. Figure 3 shows a sucrose gradient pattern of ribosomes obtained with control rats and rats given IP 5 mg F (Solu-Cortef, Upjohn Co., Mich.) 4 hr prior to sacrifice. Administration of the glucocorticoid resulted in a decrease in the heavy polyribosome fraction of the gradient and a slight decrease in the lighter ribosome species. The influence of prefeeding weaning rats for 3 days prior to hydrocortisone administration was also studied, and these results are shown in Figure 4. Here again it is noted that a reduction occurred in the heavy polyribosome region of the gradient and also of the lighter ribosome species.

Two units of insulin given IP 2 hr prior to sacrifice consistently resulted in an increased yield and proportion of the larger aggregates as compared with saline-injected controls (Figure 5A)

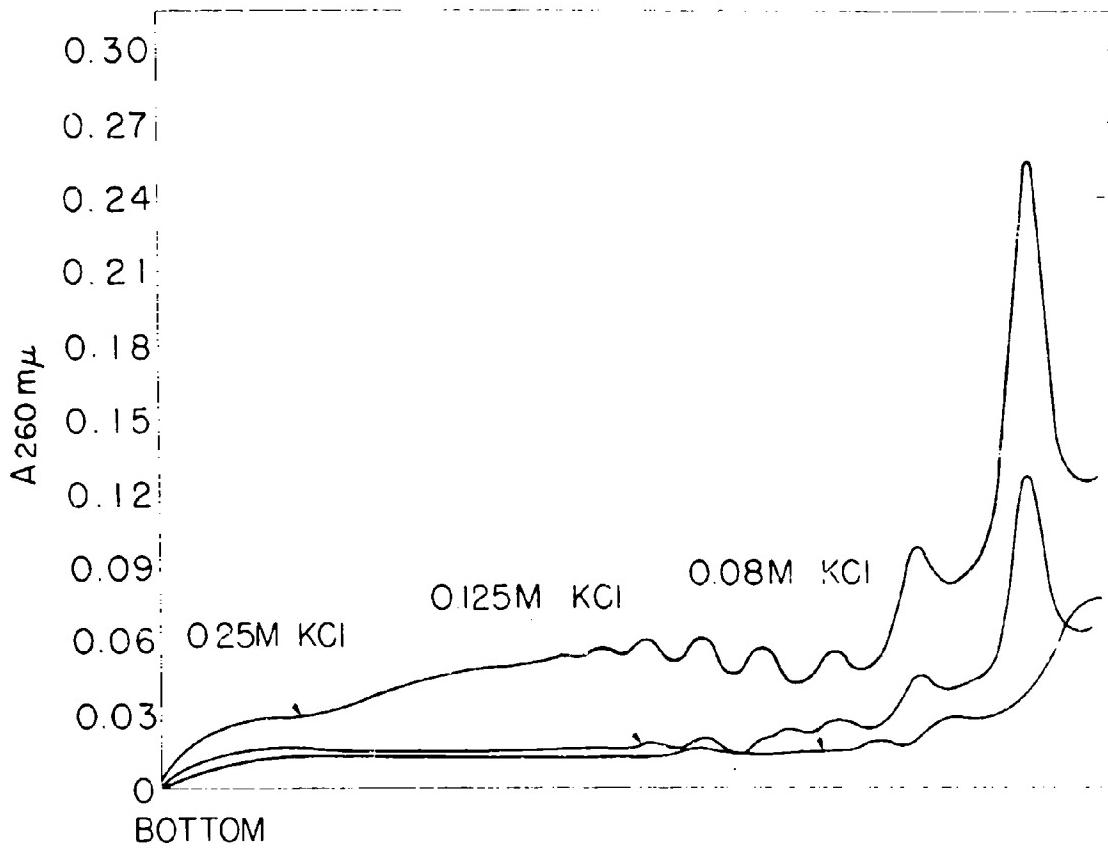


FIGURE I. SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMES FROM A POST-MITOCHONDRIAL SUPER-NATANT OF RAT SKELETAL MUSCLE.

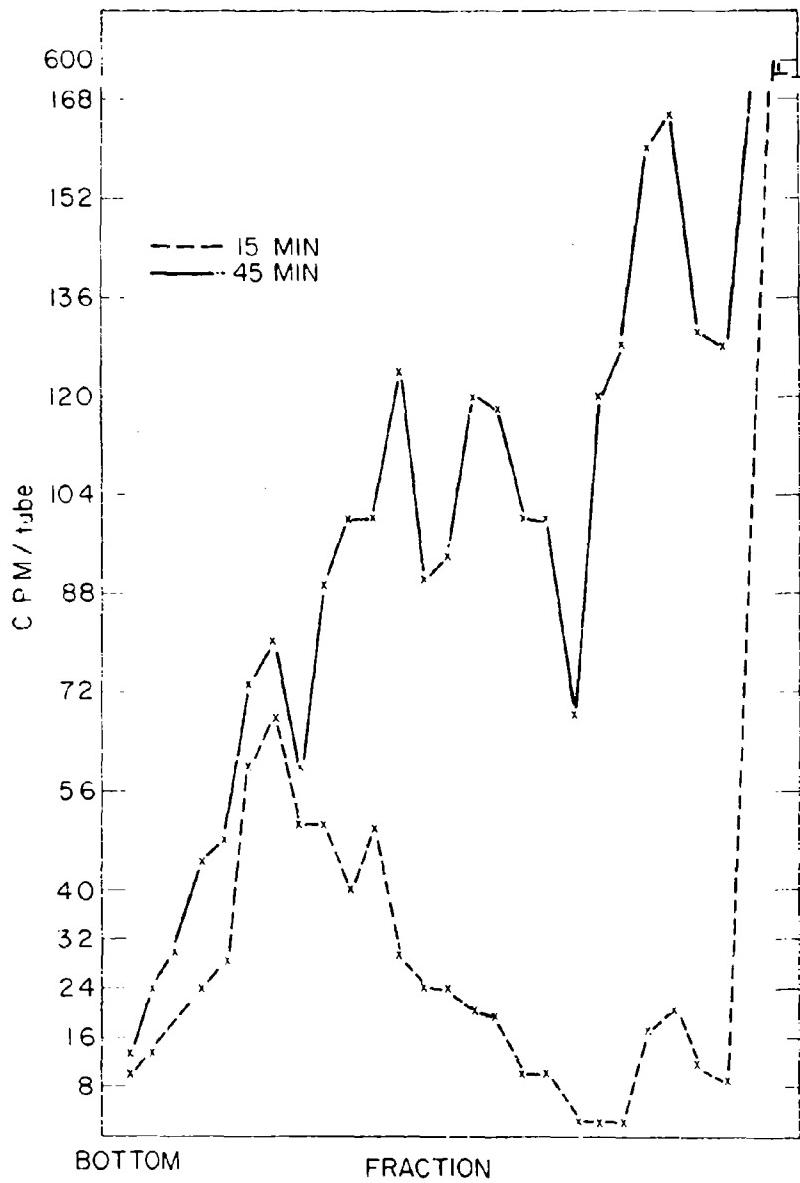


FIGURE 2. DISTRIBUTION OF PROTEIN RADIOACTIVITY IN FRACTIONS OBTAINED FROM A LINEAR 15-40% SUCROSE DENSITY GRADIENT OF RAT SKELETAL MUSCLE RIBOSOMES.

The influence of nutritional status was also evaluated by examination of the response to insulin in protein-depleted and starved rats. As shown in Figure 5B, prefeeding weanling rats with a protein-free diet for 3 days prior to injection did not markedly change the pattern of response to this hormone. However, in contrast to these results, the same dose of insulin administered to rats starved for 52 hr did not bring about an increase in the absorbance in the heavy polyribosome region of the gradient. These results are shown in Figure 5C.

The profiles shown in Figures 5B and C also demonstrate the effects of protein-free feeding and starvation since both of these dietary treatments caused a reduced level of the large ribosomal aggregates.

Some of these changes in ribosome aggregation may be a reflection of changes in the ribonuclease activity of skeletal muscle¹⁰; we have not ruled out this possibility. However, Buchanan and Schwartz¹⁰ recently suggested that muscle protein metabolism was not influenced by lysosomal enzyme activity, at least, following F treatment.

We have not examined the effects of these hormonal treatments on the activity of muscle ribosomes, this being an area for future investigation, particularly the influence of F on muscle ribosomes. However, recent studies by other workers^{11,12} have revealed good correlation between the aggregation of muscle ribosomes and their ability to catalyze protein synthesis *in vitro*. Although our results on the changes in ribosome aggregation were in accord with the established net effects of both hormones and diet on muscle protein content, the mechanism(s) associated with these changes were not demonstrated. Currently we are examining the effects of a low protein intake during a relatively prolonged period on muscle ribosomes. Although the results are preliminary, it appears that after 20 days of feeding a 3% casein diet, the distribution of ribosomes species is not markedly different from *ad libitum*-fed controls given an 18% casein diet. However, the *in vitro* activity of muscle ribosomes obtained from the low-protein group is substantially lower than the activity found with ribosomes of the control rats.

With this brief background in mind, we have begun to examine the effects of *S. typhimurium* infection on muscle ribosomes. In Figure 6 the aggregation of muscle ribosomes 1, 2, 3 and 5 days postinfection is shown and compared with profiles obtained from pair-fed noninfected control rats. During the first 2 days, lowered levels of heavy polyribosomes and higher levels of the monomer and dimer species are seen in infected animals. By the 3rd day, the difference between infected and noninfected was less marked; by day 5 the distribution of ribosomes was approximately the same in both groups.

The *in vivo* incorporation of C^{14} -leucine into nascent peptides of muscle ribosomes, measured 3 hr after an IP injection of the labeled amino acid, was also studied in this experiment. The results obtained

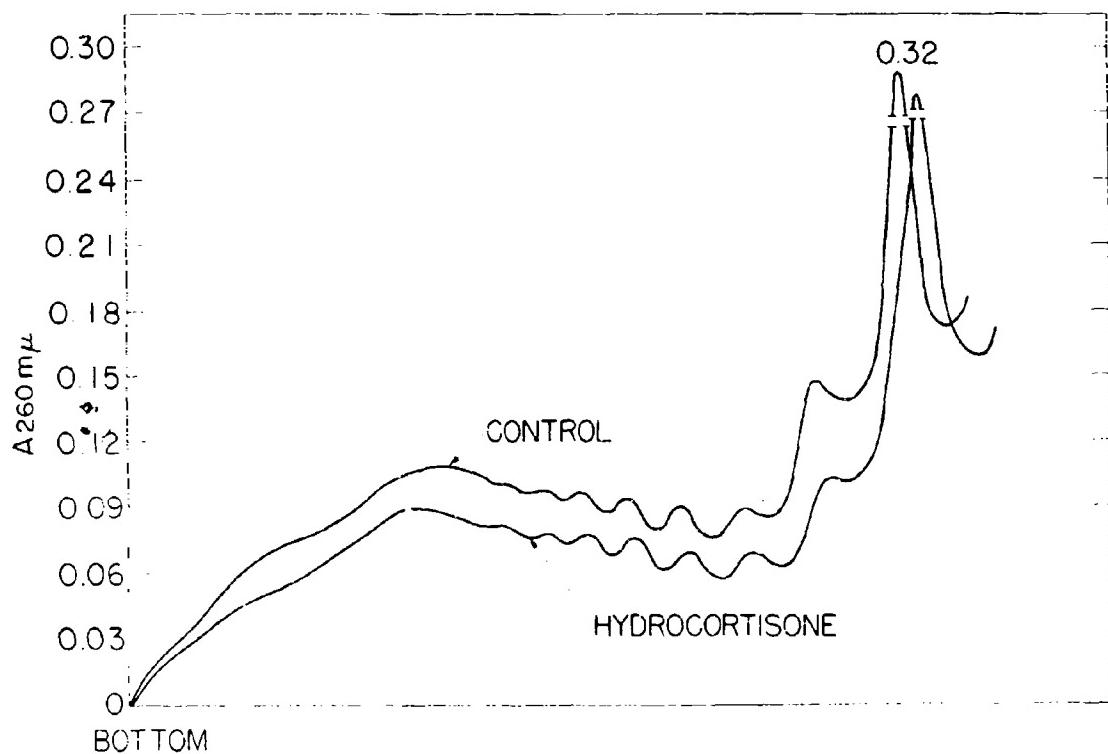


FIGURE 3. EFFECT OF HYDROCORTISONE ON RAT SKELETAL MUSCLE RIBOSOMES.

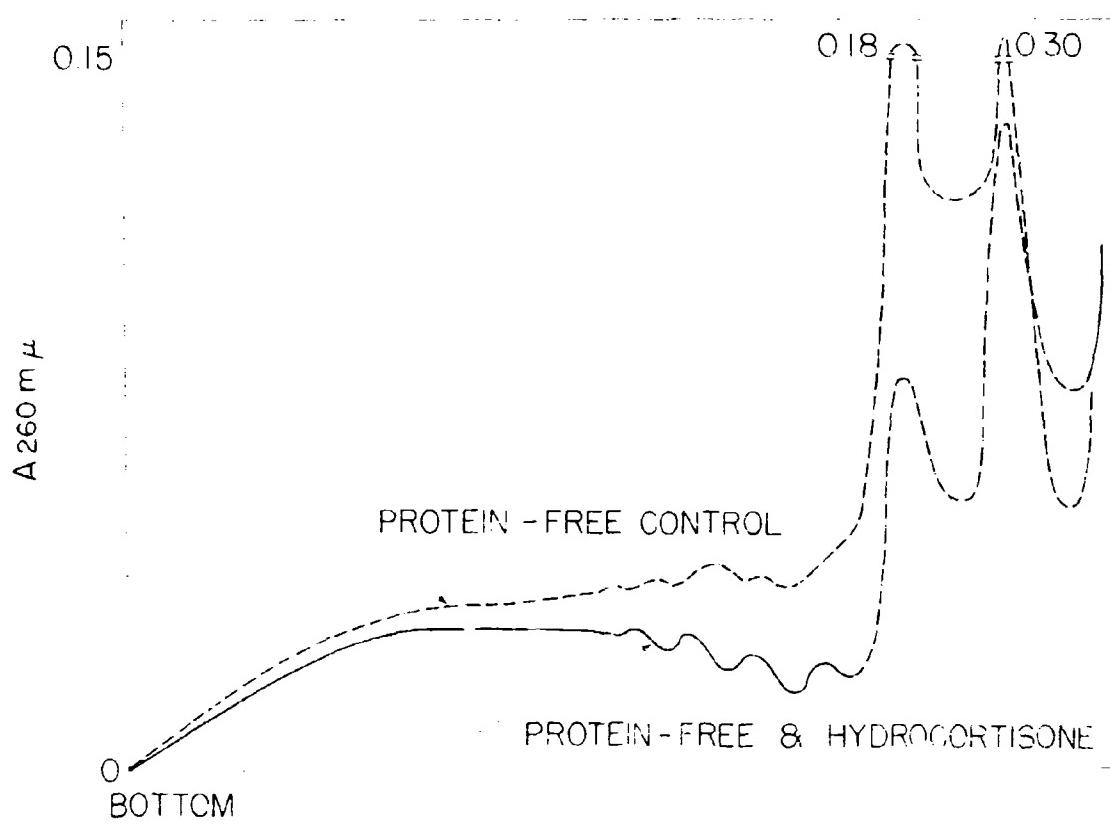


FIGURE 4. EFFECT OF HYDROCORTISONE ON SKELETAL MUSCLE RIBOSOMES IN RATS FED A PROTEIN-FREE DIET.

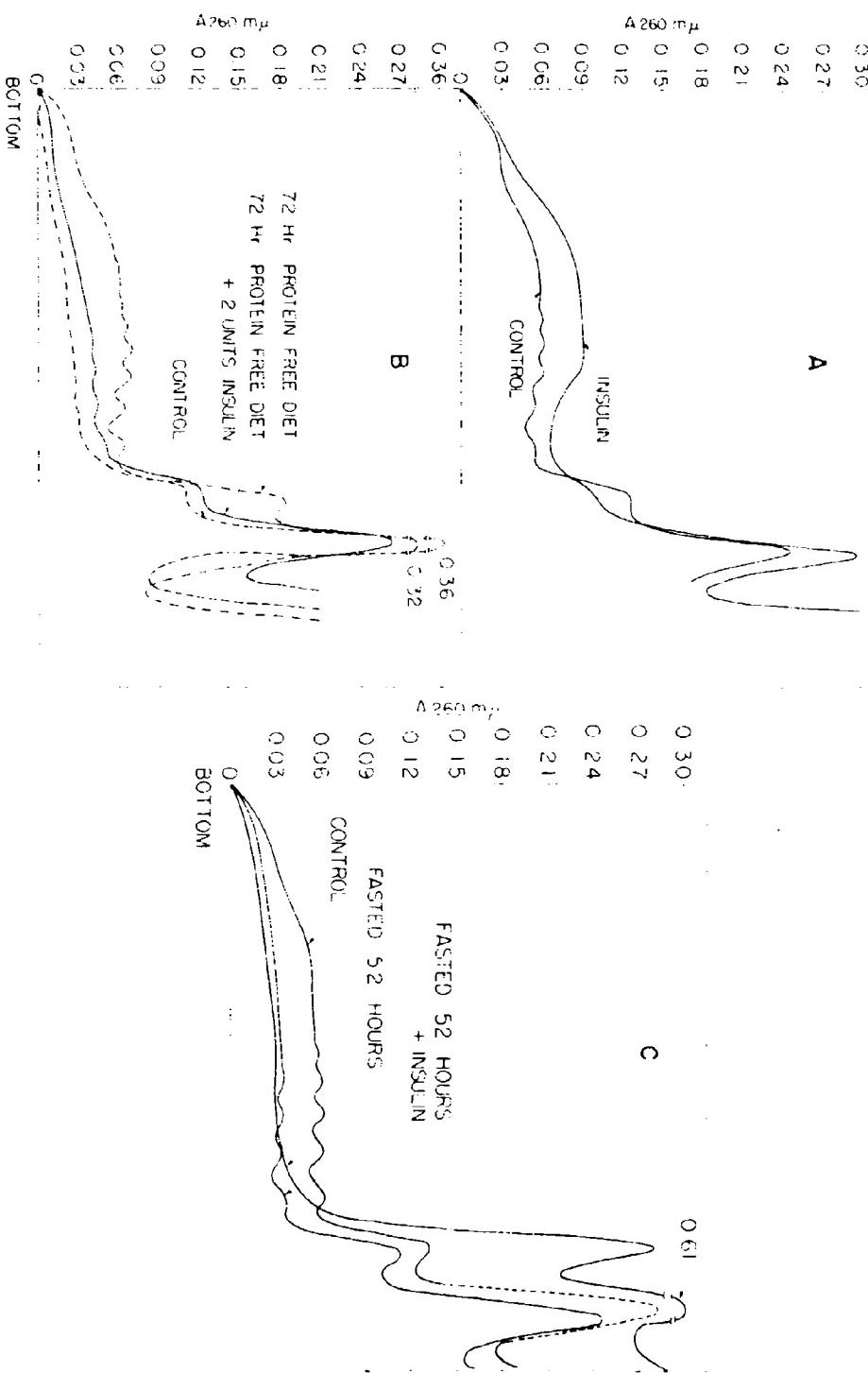


FIGURE 5. EFFECT OF INSULIN ON SKELETAL MUSCLE RIBOSOMES OF RATS. A. INSULIN ALONE. B. PROTEIN-FREE DIET. C. STARVATION.

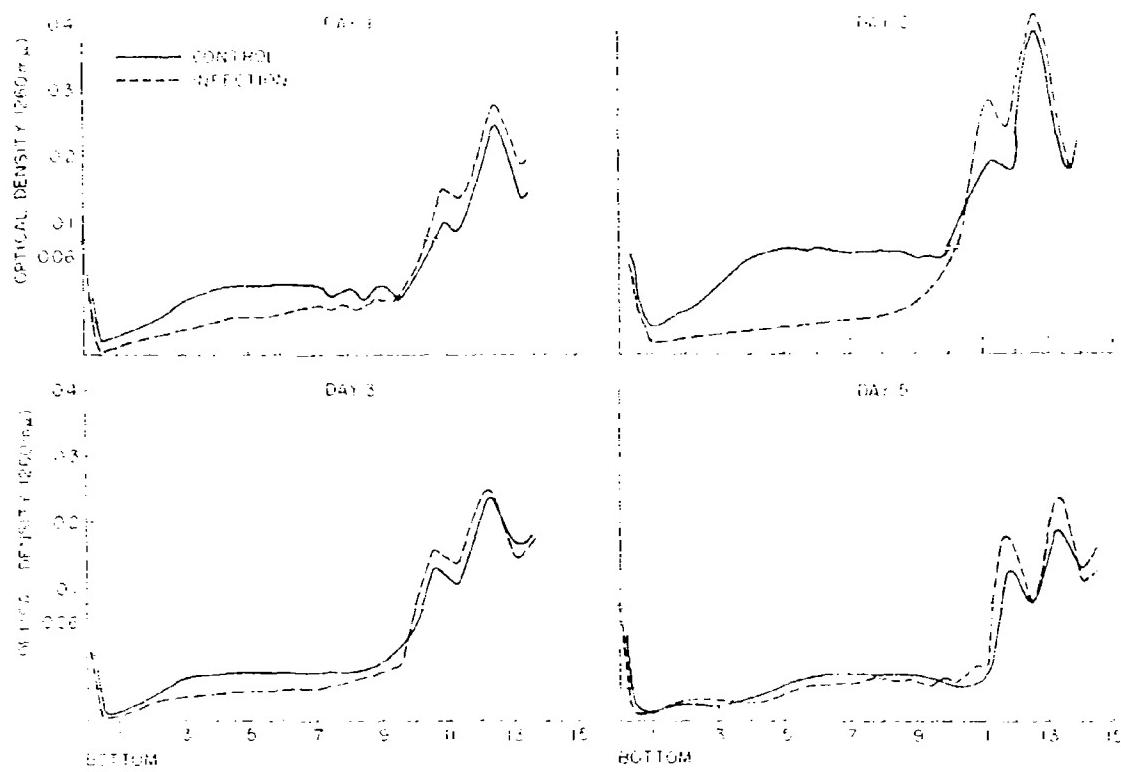


FIGURE 6. EFFECT OF S. TYPHIMURIUM INFECTION IN THE RAT ON THE SEDIMENTATION OF MUSCLE RIBOSOMES.

are shown in Figure 7A. During the first 2 postinjection days, it appears as though the activity of the ribosomes is reduced; these findings correlate with reduced levels of the heavy aggregates shown in Figure 6. Between days 3 and 5, postinfection incorporation of the label returned toward normal values, it being about 75% of the noninfected control value by the 5th day.

In a second experiment the in vivo and in vitro uptake of label of muscle ribosomes was studied in rats given adequate or low protein diets prior to infection. The results obtained for the in vivo incorporation of C^{14} -leucine by muscle ribosomes are shown in Figure 7B. As in the first experiment, the uptake of radioactivity was reduced during the first 2 days after infection in both dietary groups, and the pattern of response during this period appears to be similar for rats prefed low or adequate protein diets. A tendency toward an increased level of incorporation on day 3 was shown by the well nourished group but not in rats given the low protein diet.

The synthetic capacity of ribosomes was also treated in vitro during this experiment and these results are shown in Figure 8. Ribosomes prepared from infected rats given the low protein diet showed a reduced capacity for amino acid incorporation, and this reduction was maintained throughout the 7-day period of study. For rats given an adequate protein diet, the in vitro capacity of ribosomes was reduced during the first 2 days after infection but tended to return toward the preinfection level on day 3; by day 7 normal values were obtained. Therefore, during the first 2 or 3 days, the response of muscle ribosomes was similar in both dietary groups but following this time responses differed.

The cumulative deaths which occurred in rats infected with S. typhimurium in both experiments are shown in Table II. In accordance with previous findings^{1,13}, rats prefed a low protein diet showed a lower resistance to infection than did adequately fed rats.

The uptake of labeled amino acid into mixed liver protein following infection of rats given low or adequate protein diets is shown in Figure 9. The pattern of response was similar in both dietary groups, but the magnitude of change was greater in the well nourished rats. During the first 3 days after infection, the uptake of labeled amino acid into mixed liver protein of rats given the 18% casein diet remained at a higher level than that obtained before infection. However, by day 3, in rats given a low protein diet, the uptake of label was slightly lower than the value obtained before infection in this group. Presumably, these results reflect the changes which have been found to occur in the activity of the microsomal fraction of liver following bacterial infection.¹⁴ It is interesting to note that these increases occur at a time when the protein synthesis capacity of muscle ribosomes is reduced. This pattern of response is similar to that produced in rats treated with glucocorticoids;⁴ our findings also show that it results in a reduced level of heavy ribosome

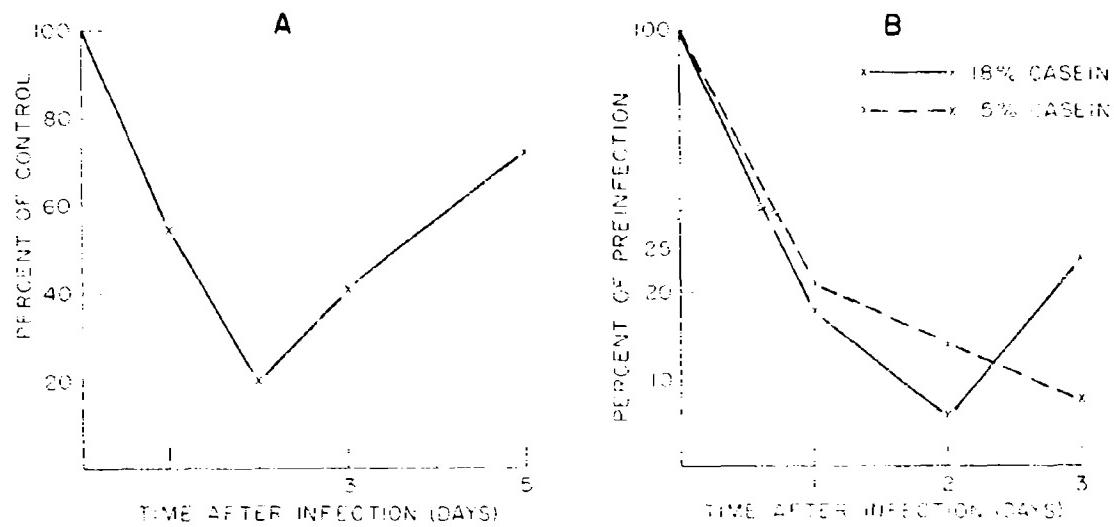


FIGURE 7. EFFECT OF *S. TYPHIMURIUM* INFECTION ON
IN VIVO UPTAKE OF C^{14} -LEUCINE INTO NASCENT
PEPTIDES OF MUSCLE RIBOSOMES. A. % OF
CONTROL POOLED VALUES. B. % OF PREINFECTION
VALUES.

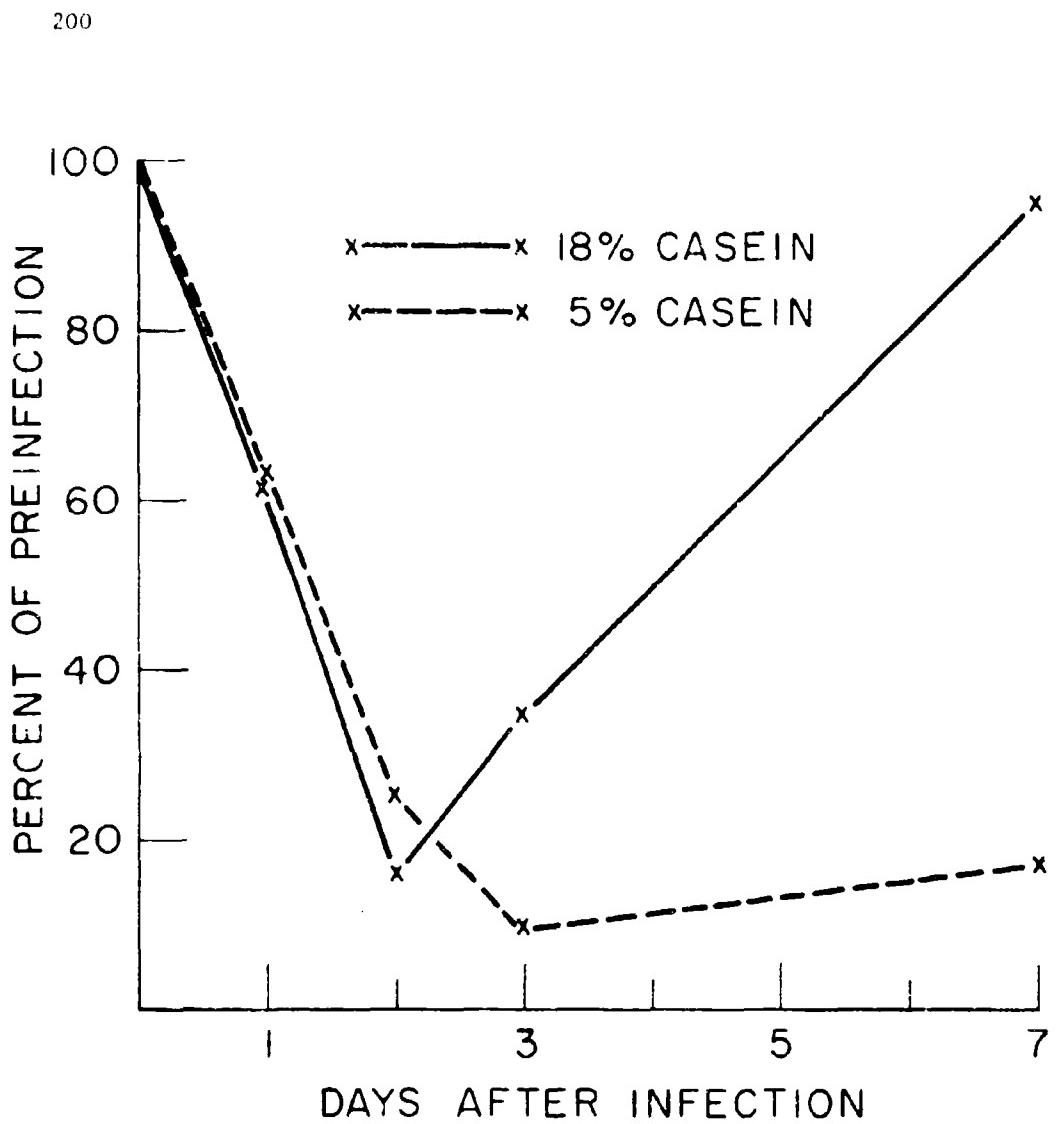


FIGURE 8. IN VITRO ACTIVITY OF MUSCLE RIBOSOMES PREPARED FROM RATS FED AT 2 LEVELS OF PROTEIN INTAKE FOLLOWING INFECTION WITH *S. TYPHIMURIUM*.

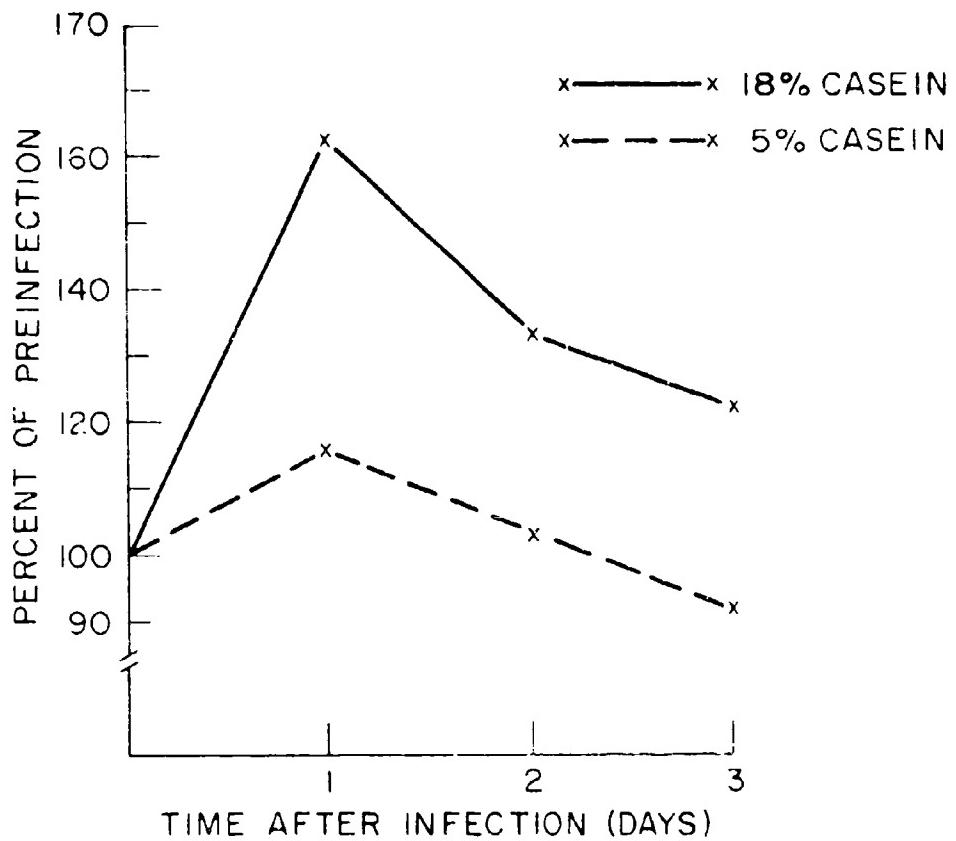


FIGURE 9. EFFECT OF S. TYPHIMURIUM INFECTION ON C¹⁴-LEUCINE UPTAKE INTO MIXED LIVER PROTEIN.

TABLE II. DEATHS IN RATS FED 2 DIETS FOLLOWING SALMONELLA TYPHIMURIUM INFECTION

EXPERIMENT NO.	GROUP	NO. INFECTED	CUMULATIVE DEATHS BY DAYS				
			1	2	3	5	7
1	18-C-1	20	0	0	0	0	0
	18-I-1	20	0	0	0	1	3
	18-I-1b	20	0	0	1	1	5
2	5-I-2	80	9	19	32	34	37
	18-I-2	80	1	2	14	18	20

aggregates in muscle tissue. However, the decreased level of polyribosomes following infection appeared to be more marked than the decrease we observed following injection of 5 mg F; this suggests that other factors are responsible for the changes which we have observed during the stress of infection. Furthermore, our results do not establish whether the experimental treatments which we have studied exert a direct or indirect effect on muscle ribosomes. This would be difficult to establish in the intact animal, but the objective of the present studies is to evaluate the host-metabolic response to infectious stress.

The nature of the change in the synthetic capacity of muscle ribosomes following S. typhimurium infection requires further study. Our experiments are only a beginning and expose the need for considerable further investigation. The activities of ribosomes prepared from various regions of the sucrose gradients should be examined in an attempt to establish whether the polyribosome itself is less active following infection. Parallel hormonal studies, particularly with glucocorticoids, are needed to allow improved correlations to be drawn on the responses of muscle to infection and hormonal balance. The function of ribosome proteins is not well understood, and it is possible that these may play important regulatory roles and may be susceptible to hormone changes produced by an infection.

So far as our own studies are concerned, we appreciate the need to standardize more precisely during the stress response the intensity of the infection and the timing of the ensuing course of events. Furthermore, we have concentrated on a limited aspect of muscle protein metabolism, and the other steps in protein synthesis should be examined. The catabolic aspects of muscle protein metabolism should also be studies with an eventual view to defining the relationships of the overall protein catabolic response of infection, protein status, and muscle metabolism.

SUMMARY

In rats given adequate or low protein diets, reduced in vivo and in vitro uptake of C^{14} -labeled amino acid into nascent peptides of ribosomes occurred during the first 3 or 5 days of study following S. typhimurium infection. The ratio of polyribosomes/total ribosomes decreased after infection, and these changes were related to the in vivo C^{14} -incorporation by muscle ribosomes. Rats given a low protein diet died sooner than adequately fed rats, but this difference was not strongly reflected by differences in muscle ribosome response in the 2 dietary groups.

The sedimentation of muscle ribosomes in sucrose gradients was studied in intact rats treated with F and insulin and in rats maintained under different dietary conditions. A low protein intake for 3 days, starvation for 32 or 52 hr, or 5 mg F given 4 hr before sacrifice caused reduced levels of polyribosomes. Insulin administration increased the levels of polyribosomes. The decreased polyribosome levels following infection appeared to be more marked than that following F administration. Reduced food intake following infection may be an important factor associated with the decreased muscle polyribosomes.

The turnover of muscle ribosomal RNA was measured in rats given adequate or low protein intakes. No significant difference was observed in these 2 dietary groups.

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DISCUSSION OF SECTION IV

Sidney H. Ingbar, M.D.*

I would like to confine my comments to the certain aspects of endocrine response of the whole organism to infection. The situation is complex because a number of different questions are involved. The first question has been touched upon already, and is, "How much of the metabolic and endocrine response to infection is due to the infection per se, i.e., to various specific toxins, fever, etc., and how much, to the general stress reaction?" Which are adaptive responses on the part of the host and which are merely a reflection of disease in the host? The second question which we ultimately have to deal with is, "Which metabolic responses are beneficial to survival and which, detrimental?" It is of great interest that protein synthesis and anabolic aspects of metabolism may be enhanced in the infected animal, as we have heard. One may assume that these changes have some survival value, but whether this is truly the case and the precise manner in which they do have some effect is of course unknown.

Another problem in dealing with this general area arise from the complexity of the endocrine system, with feedback mechanisms operating at multiple levels, not only with regard to the individual endocrine organs but to interactions between several endocrine organs in a multiplicity of ways. For example, as Dr. Shambaugh pointed out, we know that changes in the availability or the activity of thyroid hormones alter the rate of degradation of adrenocortical steroids. Such changes also may influence the responsiveness of tissues to these steroids. In view of the recent appreciation of the anabolic actions of thyroid hormones, it is entirely possible (and I think that the enzyme studies shown would suggest that this is the case) that anabolic activities of the corticoids may depend upon the availability of adequate quantities of thyroid hormone. Another complicating factor is the permissive role which hormones may play with respect to metabolic responses; these have been touched upon by Dr. Munro. Finally, two other problems that disturb us are first, the differences between acute and prolonged responses to infection, and second, substantial species variation, which prevents extrapolation from the animal model to infection in the human.

With regard to the thyroid, for example, what little we know about the response to infection indicates that there is great species variation. The rat is different from the mouse, the mouse differs from the guinea pig, the rabbit differs in certain respects from all the others, and the human probably is different from other primates, at least in particular aspects of the thyroidal response to stress.

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Rather than discuss some of the areas that have been covered very elegantly by preceding speakers, I shall try to point out certain areas in which our knowledge is sketchy or entirely lacking and others in which what we have assumed to be the case may not, in fact, be true. The response of the adrenocortical system raises the question of how general the endocrine response to infection or other stress may be. We know that the adrenocortical response is mediated through the hypothalamus and thence the pituitary. It is reasonable to ask whether the pituitary responds to stress, including infectious stress, a nonspecific way, with generalized hypersecretion of its hormonal products. Very few data have been available on this point, particularly in man. Figure 1 depicts some evidence that has recently become available from the work of Kohler and associates.^{1/} In assessing the validity of certain clinical procedures for the diagnosis of hypopituitarism, these workers studied several aspects of the pituitary response to the administration of pyromen, a bacterial pyrogen. Plasma concentrations of luteinizing and growth hormones, both measured by immunoassay, and of cortisol, a reflection of the secretion of ACTH are shown on the left. The data reveal that while secretion of both growth hormone and ACTH is stimulated by this pyrogenic agent, secretion of luteinizing hormone is not. We have evidence, then, of some specificity of the pituitary response. Shown on the right is another interesting observation. In most circumstances, hyperglycemia is associated with suppression of growth hormone secretion, and hypoglycemia with stimulation. One observes here, however, that the response of growth hormone to pyrogen is not suppressed by sustained hyperglycemia; obviously, secretion is responding to stimuli other than the availability of glucose. Figure 2 gives further evidence of the specificity of the acute pituitary response to pyrogen administration.^{1/} Here, growth hormone (GH) responses to pyrogen are shown for 3 patients with primary myxedema, in whom measurably elevated levels of TSH are present in the plasma. Although growth hormone is increased in response to glycogen, TSH concentration, and presumably secretion, are acutely depressed. It should be emphasized that these results represent the response to a pyrogenic stress of only a few hours duration. What would happen in the more prolonged and complicated circumstances of actual infection is impossible to say, although Dr. Shambaugh has given us a number of speculations.

Next, I'd like to consider in some detail the area of thyroid hormone physiology in infection and to approach it particularly from the standpoint of changes in thyrcid hormone binding, since we know perhaps more about this than any other aspect of thyroid hormone economy in the ill patient. There are in the blood 2 major binding proteins for the thyroid hormones. These are shown in Figure 3 which demonstrate radioautographs of electrophoretograms of serum containing ^{131}I -labeled thyroxine and 3,5,3'-triiodothyronine.^{2/} Thyroxine (T4) is associated with an inter-alpha thyroxin globulin (ITG), and on the right (see arrow), with a protein which has been referred to already as the thyroxine binding prealbumin (TBPA). Albumin apparently acts as a secondary carrier. Triiodothyronine (T3), the more active and more rapidly turned over hormone, is not bound by TBPA, but is

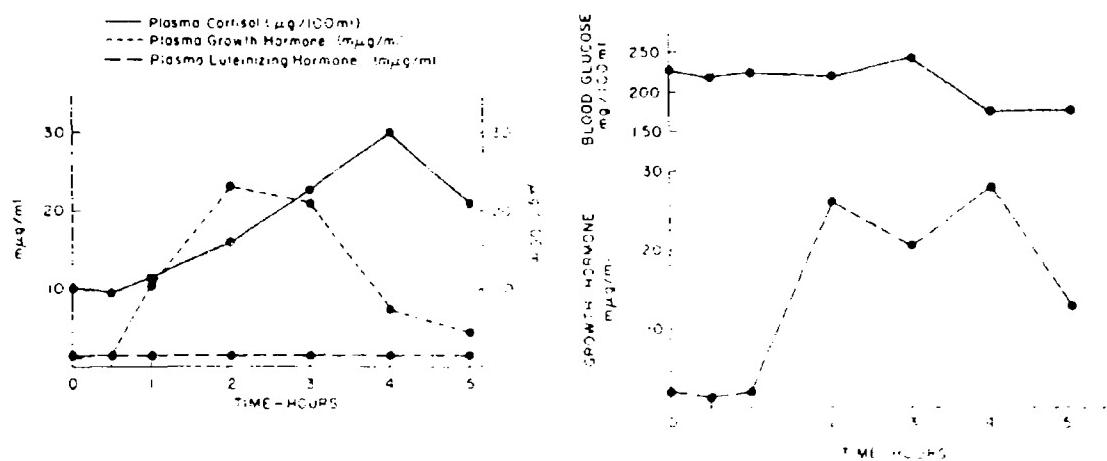


FIGURE 1. HORMONE RESPONSES TO PYROGEN IN NORMAL MAN (LEFT) AND GH RESPONSE DURING CONTINUOUS INFUSION OF 10% GLUCOSE (RIGHT). ↗ (Reprinted by permission of The Endocrine Society)

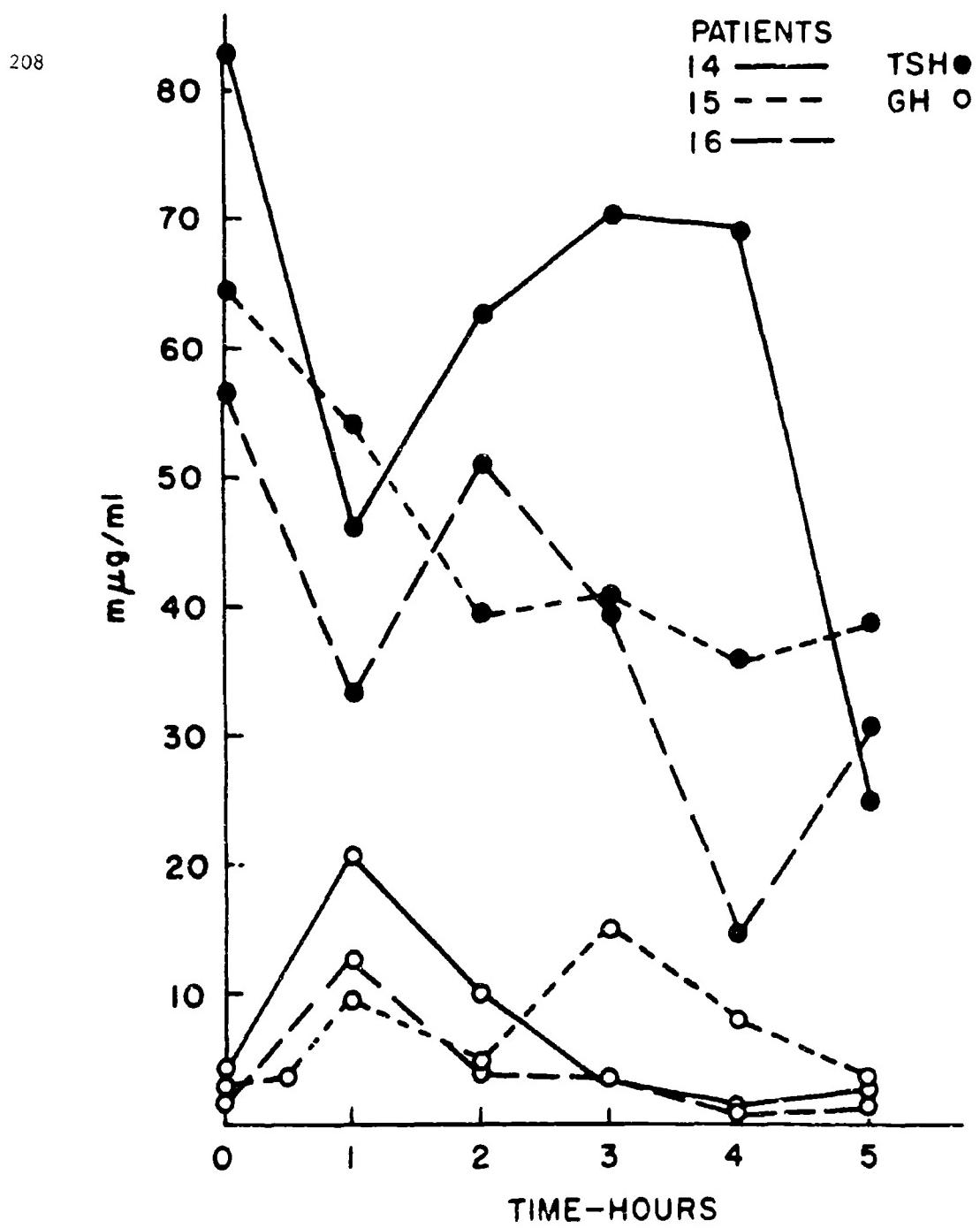


FIGURE 2. COMPARISON OF THE TSH & GH RESPONSE TO PYROGEN IN 3 PATIENTS WITH PRIMARY HYPO-THYROIDISM. (Reprinted by permission of The Endocrine Society).

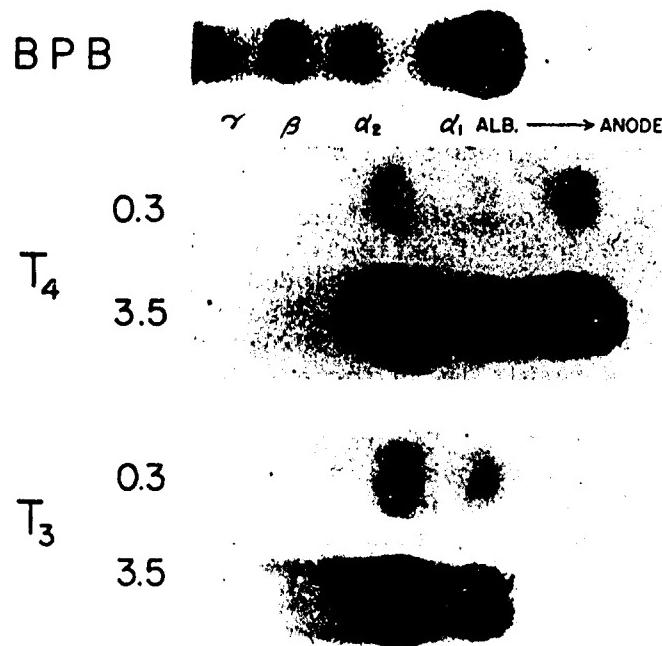


FIGURE 3. BINDING OF THYROXINE & TRIIODOTHYRONINE IN SERUM DURING ELECTROPHORESIS IN TRIS-MALEATE BUFFER. (BPB = brom-phenol blue. T_4 & T_3 = radioautographs containing 0.3 & 3.5 μ g % added hormone). (Reprinted by permission of The Endocrine Society).

bound by TBG (below). It is thought that these proteins, by binding the hormone in a sense, inactivate it metabolically. A very small proportion of the thyroid hormones is unbound or free. This moiety, unlike that which is bound, is thought to have access to the cells where it can not only initiate metabolic action, but becomes susceptible itself to degradation or excretion. A corollary of this formulation is that the proportion of free hormone would determine its fractional turnover rate and that the absolute concentration of free hormone would be an important determinant of overall hormonal effect. Under most circumstances, these homeostatic mechanisms would seek to defend the concentration of free hormone.

Several years ago we found that thyroxine-binding activity of TBPA is often decreased in the serum of patients with one of a variety of severe or moderately severe illnesses. These included vascular accidents, acute infections, trauma, and such chronic illnesses as cirrhosis and malignancy. From the postulated role of hormonal binding, one would expect that this decrease in TBPA would result in an increase in the free thyroxine fraction in the serum. With the development of methods for measuring this function, the question was investigated. Figure 4 depicts correlative studies by Oppenheimer et al.^{3/} which demonstrated a close relation between the relative saturation of TBPA and the free thyroxine concentration in the blood. Sick patients often have a very striking elevation in the proportion of free thyroxine in their blood. Figure 5 depicts studies that we carried out with a I^{131} -labeled preparation of purified TBPA to determine whether the reduction in binding capacity of prealbumin that occurs in the ill patient is due to a change in its rate of synthesis or change in the rate of degradation.^{4/} This patient received typhoid vaccine twice and developed substantial fever both times. The disappearance curve of the labeled protein is absolutely unaltered while, as shown below, the binding capacity of the protein in the serum is greatly reduced. This suggests very strongly that the decreased concentration of TBPA in the blood is due to an acute inhibition of TBPA synthesis. As a matter of fact, the binding capacity of TBPA in the blood declines with about the same half-time as that of the labeled protein, indicating that synthesis is shut off quite abruptly. It would be important to find out the mechanism by which fever, illness, or operation initiate this reduction in TBPA synthesis. The change is certainly in contrast to the apparent enhancement of hepatic protein synthesis induced by stress that has already been discussed. Quite clearly this change is not due to cortisol; in fact, large doses of adrenal glucocorticoids increase the concentration of TBPA in the blood. Be that as it may, one would assume that this decrease in TBPA synthesis should ultimately make more thyroid hormone available to the cells.

It then remained to be shown whether the postulated increase in the flux of hormone to tissues really does occur under conditions of febrile stress. Gregerman and Solomon^{5/} have shown this for patients with pneumonia. In Figure 6 is shown the disappearance slope for thyroxine in a patient with pneumonia.^{5/} The half-time of thyroxine was 1.3 days, compared to the normal of about 6.7 days. Interestingly enough, this striking acceleration

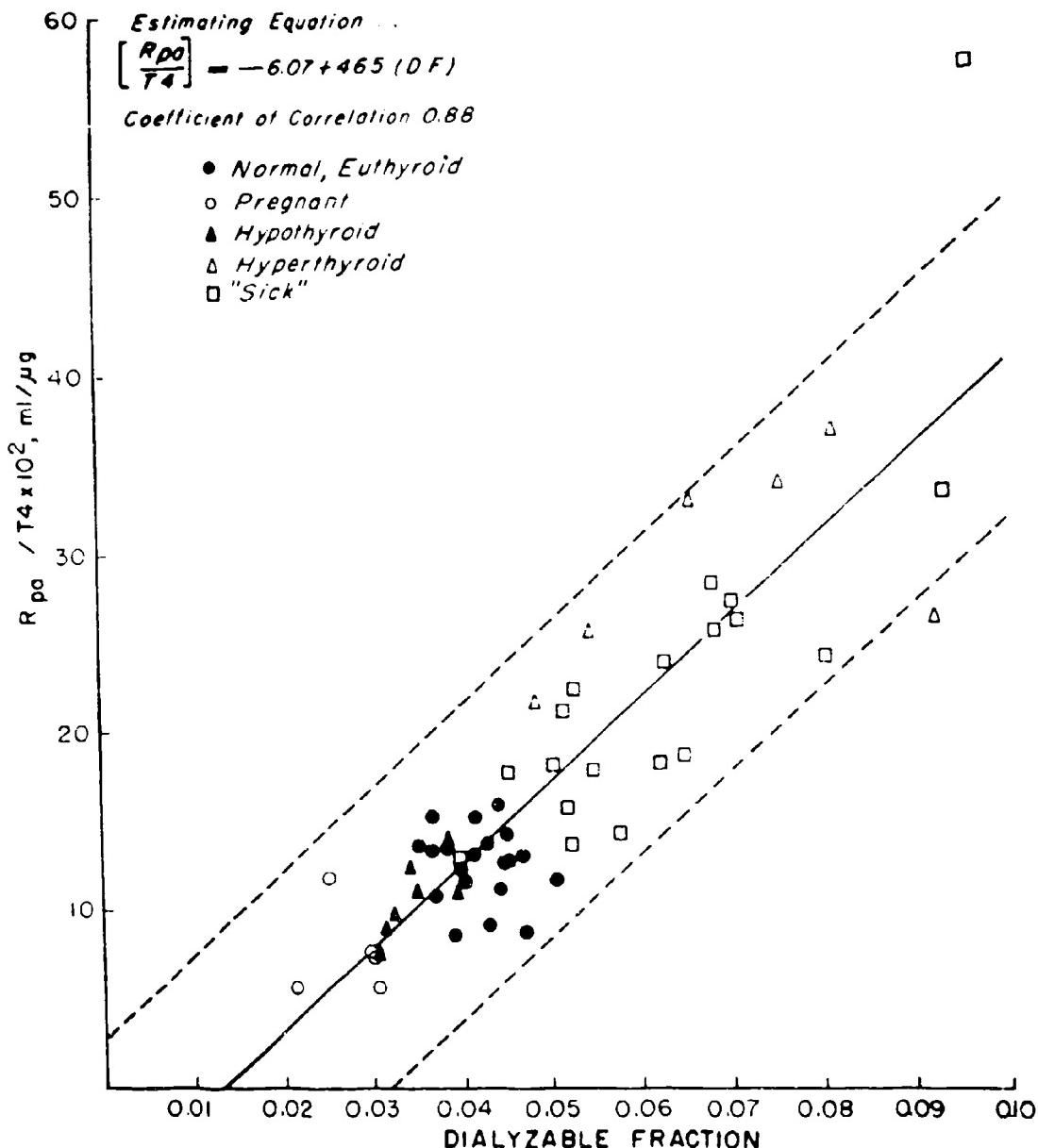


FIGURE 4. CORRELATION BETWEEN RELATIVE SATURATION OF PREALBUMIN (R_{pa}) AND FREE THYROXINE (T₄).^{3/} (Reprinted by permission of the American Society for Clinical Investigation, Inc.).

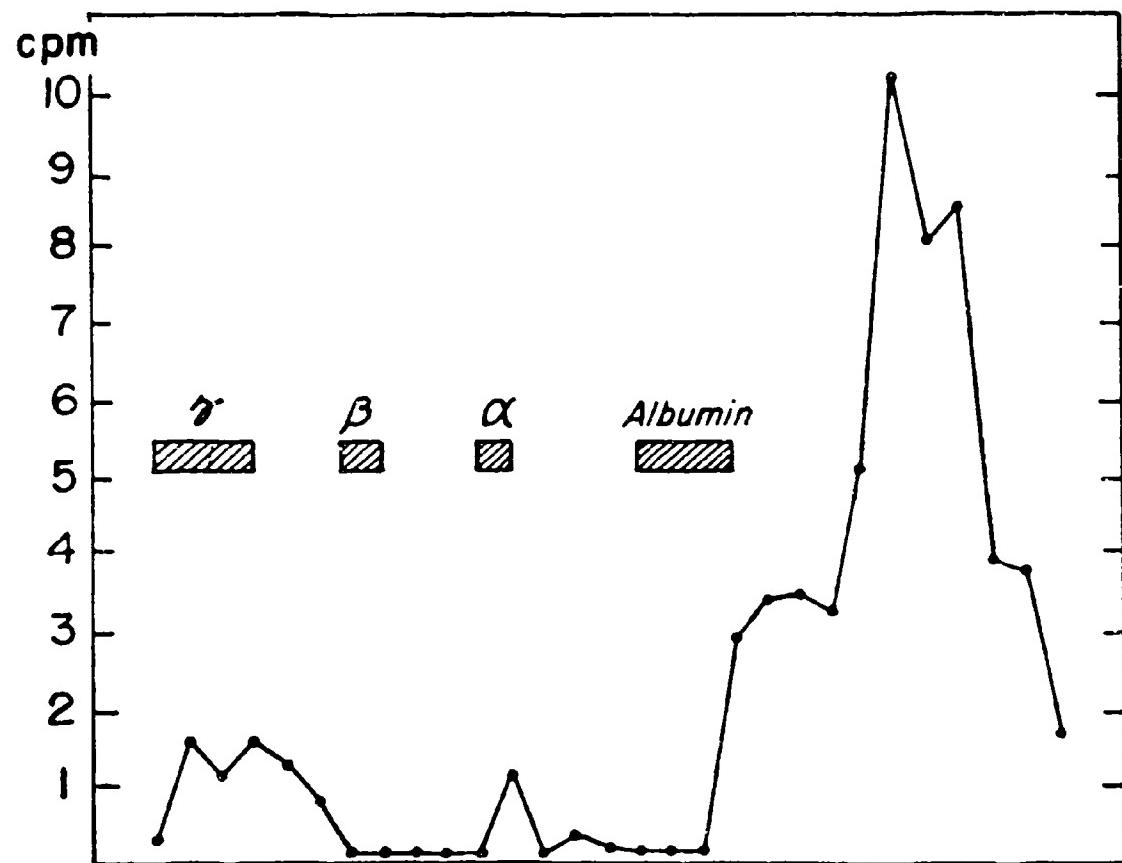


FIGURE 5. ELECTROPHORETIC MIGRATION OF PREVIOUSLY ADMINISTERED I^{131} -LABELED TBPA IN THE SERUM OF NORMAL PATIENT GIVEN TYPHOID VACCINE.⁴

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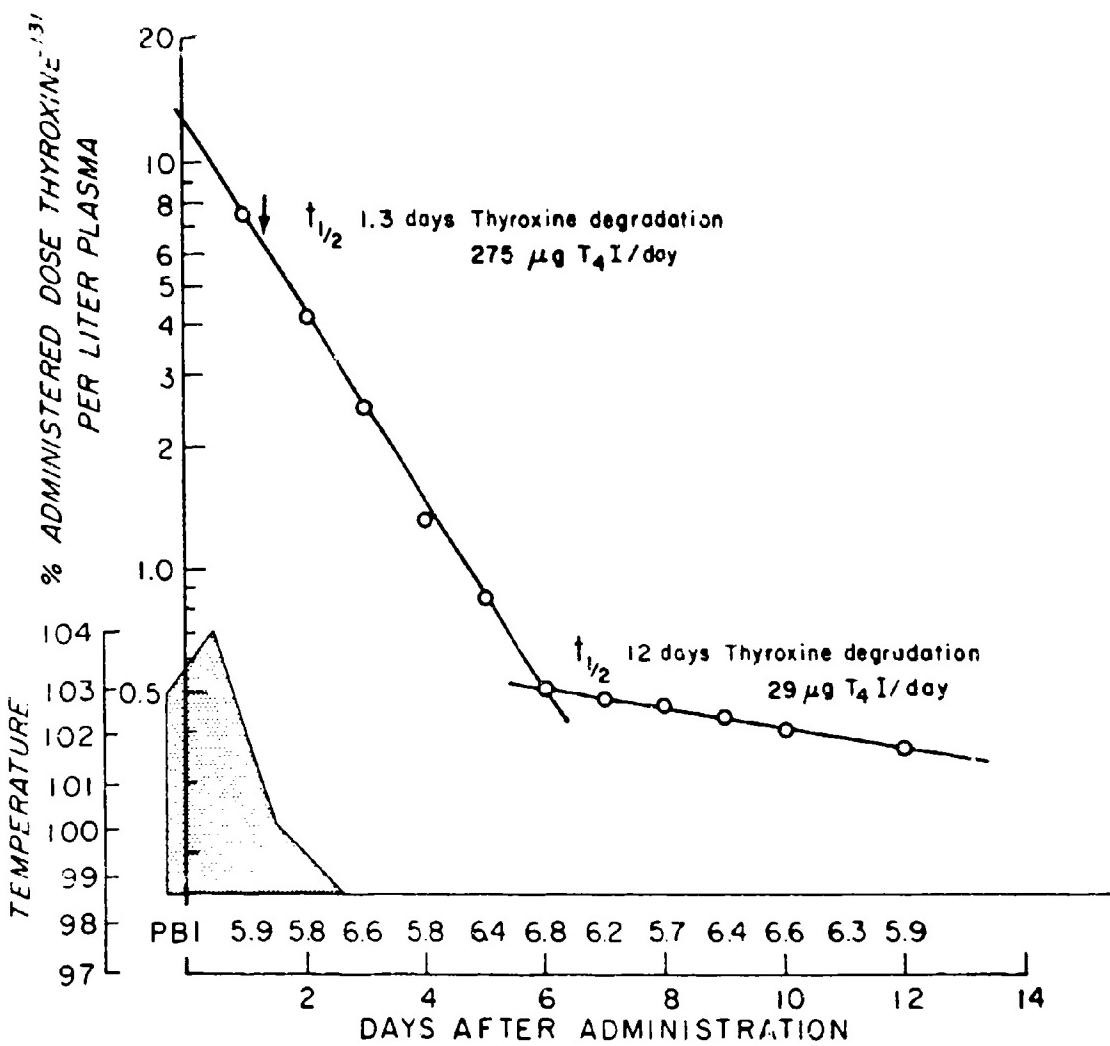


FIGURE 6. THYROXINE (T₄)-I¹³¹ TURNOVER IN PATIENT WITH PNEUMOCOCCAL PNEUMONIA, COMPLICATED BY CONGESTIVE HEART FAILURE. PBI STABLE THROUGHOUT. ^{5/} (Reprinted by permission of The Endocrine Society).

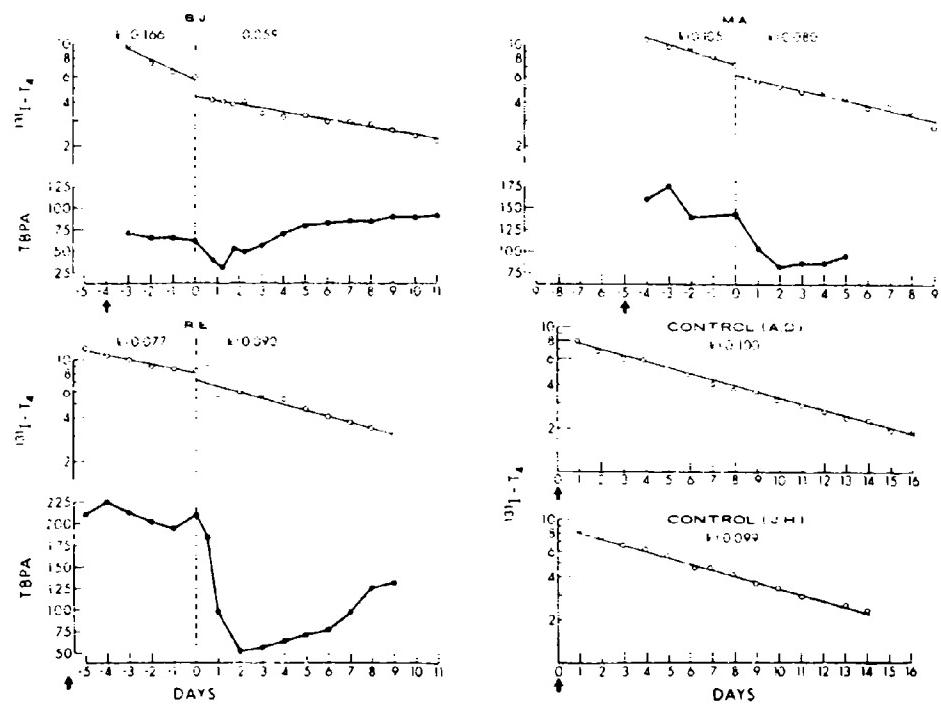


FIGURE 7. EFFECT OF SURGICAL TRAUMA ON TBPA ($\mu\text{g T4}/100 \text{ ml serum}$) AND DISAPPEARANCE OF I^{131} -LABELED T4 (% Injected I^{131} -T4/L plasma.) k = fractional removal rate (day^{-1}). $\frac{\text{d}}{\text{d}t}$ (Reprinted by permission of The Endocrine Society).

of thyroxine turnover rate continued beyond the febrile period. The second interesting point perhaps relates in a general way to some of the other phenomena that we've heard about this morning. Not only was there a temporal dissociation between the fever and the acceleration of thyroxine turnover, but after the fever had subsided, there occurred an abrupt and marked slowing of thyroxine turnover, the half-time increasing to 12 days. This rebound has been seen in other situations. We noted it some years ago in a group of soldiers exposed to cold stress; they displayed an acceleration of thyroid hormone removal during the cold and then a prolonged retardation of the half-time after they were brought back to a normal environmental temperature. We don't understand this retardation, but it has a general ring of similarity to the persistence of other metabolic alterations beyond the period of fever, about which we heard earlier.

One point which should be made in regard to all studies which deal with the disposal of thyroxine (and I presume of other hormones too) is that we have no idea whether such increased disposal represents a degradative or detoxifying disposal, or whether it indicates an enhanced passage of thyroid hormone into sites where metabolic action may be initiated. In other words, we are not certain whether accelerated disposal of thyroxine indicates that the tissues are getting more or less thyroid hormone in the metabolic sense. In the case of infectious stress we can't look at traditional indices, such as the BMR, for evidence on this point, since they may be altered by fever, per se. Rather, we should examine some system which is quite specifically thyroid hormone-dependent. One which I might suggest is the hepatic microsomal enzyme, glycerophosphate dehydrogenase. Among a large number of hormonal factors, only thyroid hormone has been shown to influence the activity of this enzyme.

From the foregoing findings it would seem logical to suggest that the decrease in TBPA induced by illness is the factor which leads to an increased proportion of free thyroxine in plasma, and that this, in turn, is at least partly responsible for the accelerated peripheral turnover of the hormone. However, as I initially indicated, certain correlations such as these lose strength when examined more closely. Recently, Dr. Kenneth Woeber in our laboratory has been able to remove TBPA from serum completely by means of a rabbit γ -globulin containing an antibody against TBPA. He found that this removal resulted in a slight increase in the free thyroxine fraction. However, the increase was smaller than we had anticipated, and the resulting values were substantially lower than those seen in both his own and other studies in sera of ill patients. This indicates that a decrease in TBPA cannot account for the increased free thyroxine fraction in the serum of the ill patient; another factor or factors, as yet unknown, must also contribute.

An additional line of evidence also tends to disrupt the foregoing chain of apparent cause and effect relationships. Studies recently reported by Bernstein et al.^{6/} are shown in part in Figure 7. Here are plotted values for labeled thyroxine disappearance from plasma and the thyroxine-binding

capacity of TBPA before and after elective surgery. In the postoperative state, there is an actual slowing of thyroxine turnover despite a marked decrease in TBPA in some patients. These findings indicate that we cannot equate surgical stress with infections or febrile stress, at least as regards their effects on thyroid hormone economy. Such complications increase the extent of effort required for understanding, but also increase its fascination.

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SECTION V

INFECTION AND GENERALIZED HOST RESPONSES

WHOLE BODY RESPONSES

MODERATOR: Dr. Leighton E. Cluff

DISCUSSANT: Dr. Charles L. Wisseman, Jr.

THE EFFECT OF HYPERTHERMIA ON PROTEIN METABOLISM IN VIVO
AND IN VITRO AS OBSERVED IN THE NEW ZEALAND WHITE RABBIT

Irving Gray, Ph.D., and Salvatore Leto, Ph.D.*

An altered metabolic state during the febrile period in the disease state has long been recognized.¹⁻⁶ Marked changes have been noted in carbohydrate, electrolyte, endocrines, and protein metabolism by these investigators. The effect of infection on nitrogen metabolism has been investigated and has been shown to cause a shift toward a negative balance. Lusk² provided an extensive analytical review of the literature on nitrogen balance in fever. From the studies of Dubois and his associates^{1,2} and others, it became evident that the degree and duration of the destruction of protein seemed to vary with the severity of the injury or disease. In early observations, the term applied to this destructive phenomenon, as seen in previously healthy individuals during the acute phases of the infection, was "toxic destruction of protein." Furthermore, this toxic destruction was found to persist long after a patient has appeared to be cured. Attempts to prevent this destruction by administering excess protein or calories, or both, could reduce, but not prevent, N loss in the acute phases. From this, Peters³ concluded that "neither accelerated oxidations nor fever can explain the uneconomical expenditure of protein, after fractures, and during convalescence from acute infections." It would, therefore, seem that other mechanisms must be looked for to explain this toxic destruction of protein in the febrile state.

Beisel^{5,6} noted that balance studies previously were initiated only after clinical illness was fully developed, or happened by chance in a patient undergoing metabolic investigation. In his investigations, normal healthy, young male volunteers were first put on a common, strictly controlled, diet and were subsequently exposed to infectious organisms. By using this method, extensive control data were obtained, as well as data which illustrated metabolic changes during the infectious illness. It was noted that both the nature of the dietary protein and the amount of intake contributed to the magnitude of N loss. As had been seen by earlier investigators,^{2,3} only a reduction, and not complete elimination of protein loss, could be achieved.⁶ Gasarov⁸ reported that fever induced by bacterial pyrogen in rabbits intensified glycogenesis and urea production from amino acids. Veselkin⁹ noted that pyrogenic fever caused an irregular increase in heat production of 20-30%, and an increased protein breakdown. Noelle,¹⁰ however, suggested that a negative N balance due to infective fever + hyperthermia, could be avoided by sufficient caloric and protein intake.

Although the negative N balance associated with infection and fever may be due to a contribution of both the organism and fever that accompanies it,

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little has been done to determine the relative contribution of each on the observed findings. Gray and French¹¹/ reported an increase in protein synthesis in mouse brain during a virus infection. Reiss¹²/ showed that overall protein catabolism was greater in infected animals, but that protein synthesis increased in liver during streptococcal pneumonia infection. The suggestion was put forward that muscle protein synthesis was impaired during infection. Recently, Lust¹³/ reported that in bacterial infection, protein synthesis is increased in the liver and small intestine, but decreased in muscle. He reported that in viral infection, overall host-protein synthesis may be depressed; liver protein synthesis was seen to be depressed initially with a return to normal. Little alteration was noted in brain or intestinal protein synthesis, while in muscle, the rate was diminished throughout the illness.

At the molecular level, Rahn¹⁴/ stated that thermal injury resulted in denaturation of cell protein. Minard and Copman¹⁵/ pointed out that at normal body temperatures, the rate of destruction of cell protein occurred slowly, while replacement by synthesis was readily maintained. A temperature exists at which the 2 rates are equal, the "optimal temperature." Further increases in temperature, because of the great disparity in the temperature coefficients of the opposing processes, lead to a rate of destruction exceeding that of synthesis. Unless the system is cooled, the cell will die, only a few degrees above the "optimal temperature"; this point is called the "thermal death point."¹⁵/ From the evidence compiled by Johnson et al.¹⁶/, Rahn's concepts have had to be modified. Studies since 1940 have established that in the reaction kinetics of isolated enzyme systems, and in more complex biological systems as well, a native, active form of protein exists in equilibrium with a reversibly inactive form. Furthermore, an irreversible process of destruction also exists. However, it is difficult to discern whether the native active form, the reversibly inactive form, or both, are acted on by this latter process. Complete reversibility, as pointed out by Johnson et al.¹⁶/, depends on brief exposure to high temperature. Since the destructive process is always present, the time element is very important.

It seems reasonable to assume that the observed changes in N metabolism during infection may be due to a contribution of both the infective organism and the fever that usually accompanies an infection, little has been done to determine the relative contribution of hyperthermia and the microorganisms to the altered protein metabolism. A preliminary study was carried out by us (Gray and Hildebrandt, unpublished data) to try to delineate the effect of elevated body temperature on protein metabolism with and without the presence of an infective organism. Chimpanzees were used. A depression of incorporation of L-methionine-S³⁵ into plasma protein was noted during hyperthermia (106 F) while in the presence of the infective organism at the same hyperthermic temperature, the incorporation level was stimulated above that seen for hyperthermia alone.

Using male, New Zealand white rabbits, we have initiated a more definitive study in an attempt to determine the effect of hyperthermia on protein metabolism. An attempt has also been made to clarify the underlying mechanism or mechanisms involved in protein metabolism in this state. In vivo experiments give only limited information about detailed biochemical mechanism by which proteins are made. However, although the elucidation of the various reactions in cellular physiology is important, we must not lose sight of the fact that we also wish to know how these reactions are modulated in the intact animal, for the benefit of the animal. Thus during this investigation, we have employed both in vivo and in vitro techniques. Preliminary findings have been reported.^{17/}

Preparation of Rabbits.--Each animal was weighed to the nearest gram. After weighing, the ears were shaved and 5 mg/kg promazine hydrochloride (Sparine^R, Wyeth Laboratories, Philadelphia, Pa.) was injected into a marginal ear vein. The animal was then fastened in a supine position on a stainless steel anatomy table, which had a large plastic blanket spread over the surface. The right femoral vein was exposed and cannulated. Two percent xylocaine was used as a local anesthetic. The sterile, silicone-coated cannula was made of polyethylene tubing, size P.E. 90. A thermistor probe was then inserted into the rectum approximately 12-15 cm and firmly fastened to the shaved tail. The rabbit was covered with a second plastic blanket from the neck down, so that it was enclosed by plastic blankets.

The blankets were connected to a K-thermia Aquamatic unit (Model RK-101, Gorman-Rupp Industries, Inc., Belleville, Ohio). Thermoregulated water was circulated by this unit through the plastic blankets. The other end of the thermistor probe was connected to an electronic control unit connected to the Aquamatic system. The control unit allows any desired rectal temperature to be set between 40 and 110 F ± 0.5. For these experiments, the control temperature was set at 100 F and the hyperthermic temperature at 105 F. Experimental periods were 48 hr.

Cutter I-5-S (5% dextrose in 0.9% NaCl), to which Sparine was added to a final concentration of 0.2 mg/ml for the hyperthermic animals and 0.3 mg/ml for the controls, was infused through each cannula. The hyperthermic animals were given 250-300 ml, controls 200 ml during the 48 hr.

All blood samples were collected via the cannula. Serum albumin and globulin were separated by the method of Pijssem and Hutchinson.^{18/} Two procedures were followed for protein determination depending on the concentration of the sample: for quantities of > 3 mg/ml of protein, the bieret method of Gerndt et al.^{19/} was used and for smaller quantities, the method of Levy et al.^{20/} as modified by Miller.^{21/}

Measurement of radioactivity was made in a Nuclear Chicago liquid scintillation spectrometer (Model 703 system). All samples were counted to an error of ± 3%.

Serum Protein Synthesis and Catabolism Studies.--For in vivo studies, the 24-48 hr period was used. At the 24th hr, S^{35} was injected into one of the marginal ear veins. Blood samples were collected at 0.5, 1, 2, 4, 8, 12, 16 and 24 hr.

For in vitro studies, animals were maintained on the pads for the 48-hr period. The animals were then sacrificed by injecting 10 ml of air via the cannula. The liver was quickly removed, placed on ice, and transferred to chilled phosphate buffer pH 7.25 ± 0.5. The liver was blotted dry, 15-20 gm were forced through a garlic press to prepare a brei. The brei, in a beaker, was kept in an ice-water bath. Ice-cold, phosphate buffer was added to the brei, in a 1:1 ratio. One μ c Me- S^{35} /gm of liver brei was added; the contents of the beaker were thoroughly agitated. An aliquot was immediately removed and homogenized for 1 min in an ice-water bath. The homogenate was centrifuged at 2 C for 10 min at 10,000 xg, the supernatant fluid was decanted and centrifuged 30 min at 30,000 xg. One-ml aliquots of the fluid were pipetted into 1 ml 20% tricholoroacetic acid (TCA) and treated as described for serum samples. The remainder of the brei suspension was incubated at 37 C for 1 hr in a Dubnoff metabolic incubator with shaking. The suspension was then quickly chilled in an ice-water bath and treated in a manner similar to the aliquot.

For in vivo degradation studies, 2.0 ml of S^{35} -labeled serum was injected into a marginal ear vein. The first 24 hr was considered a mixing period for the serum, to allow distribution of the labeled serum into the body compartments, and also, to allow the hyperthermic animals time to adjust to the new environment. Blood samples were collected at 24, 26, 28, 32, 36 and 48 hr. Degradation calculations were based on loss of serum protein specific activity.

In in vitro degradation studies, the liver was removed at 48 hr and made into a brei. One ml of labeled serum was added per 20 gm of brei. An initial aliquot was taken and the remainder was incubated for 1 hr at 37 C. Both aliquot and incubated suspension were treated as described in the procedure for in vitro incorporation.

Lysosome Isolation.--The isolation of rabbit liver lysosomes was essentially the method described by Sawant et al.²² and subsequently modified by them (personal communication). The animals were prepared as usual; after the 48-hr experimental period, the liver was removed and the lysosomes separated.

The cathepsin activity was determined as follows:

(1) The optimal pH was determined using citrate-phosphate buffer at pH 3.8, 5.0, 6.0, and 7.0.

(2) Substrate. Labeled serum protein was diluted 1:2, 1:5, 1:10, 1:20. All dilutions were placed in vials and kept frozen at -20 C. The

specific activity of the protein in the various dilutions was determined by radiometric determination of an aliquot and by protein content.^{20/}

(3) Incubation Procedure: 0.1 ml of each of the substrate dilutions was placed in a standard test tube. To each tube was added 0.9 ml of buffer. All samples were placed in an ice-water bath. One ml of a 3-X frozen and thawed subcellular liver fraction was added to the appropriate test tube. Since the majority of the activity (70-80%) was found to be associated with the supernatant fraction, this fraction was used in all subsequent enzyme activity determinations.

After addition of the liver enzymes the tubes were incubated for 30 min at 37 or 42 C in a Dubnoff metabolic incubator. The reaction was terminated by the addition of 2.0 ml of ice-cold, 20% TCA to each incubation mixture. Samples were refrigerated overnight. One drop of 1% albumin was added to each sample as a coprecipitant; all samples were centrifuged. One ml aliquots of the supernatant fluid were counted for TCA-soluble radioactivity.

(4) Co-factors: According to Beeken,^{23/} a variety of cofactors enhance cathepsin activity. Furthermore, Fruton^{24/} has indicated that cathepsins, like many enzyme systems, are activated by -SH compounds. Cathepsins B and C have been shown to require such activators. The citrate-phosphate buffers used for incubation were made with 0.04 M with l-cysteine,^{23/} as the -SH activator.

As in the preliminary experiments (Gray and Hildebrandt, unpublished data), maximal incorporation of Me-S³⁵ took place in the first 4 hr (Figure 1). It is apparent from our data that the effect of hyperthermia was to depress incorporation of labeled amino acid into plasma protein. The depression at 4 hr was about 40% below the control value. In in vivo studies, in which Me-S³⁵ incorporation into serum albumin and globulin was followed, it is seen from Figure 2 that the major effect of hyperthermia was associated with the globulin fraction.

In in vivo degradation studies serum protein specific activity during the second 24-hr period of experimental hyperthermia (mean \pm 1 SE) is shown in Figure 3. The specific activity of the first serum sample collected at 24 hr, or 0 time, was arbitrarily taken as 100%. It is apparent that hyperthermia has caused a faster rate of breakdown of serum protein than that of control animals. Table I summarizes our findings on the effect of in vivo hyperthermia on protein metabolism in vivo. It is seen that synthesis was depressed approximately 40%, while catabolism was increased about 30% in the hyperthermic state.

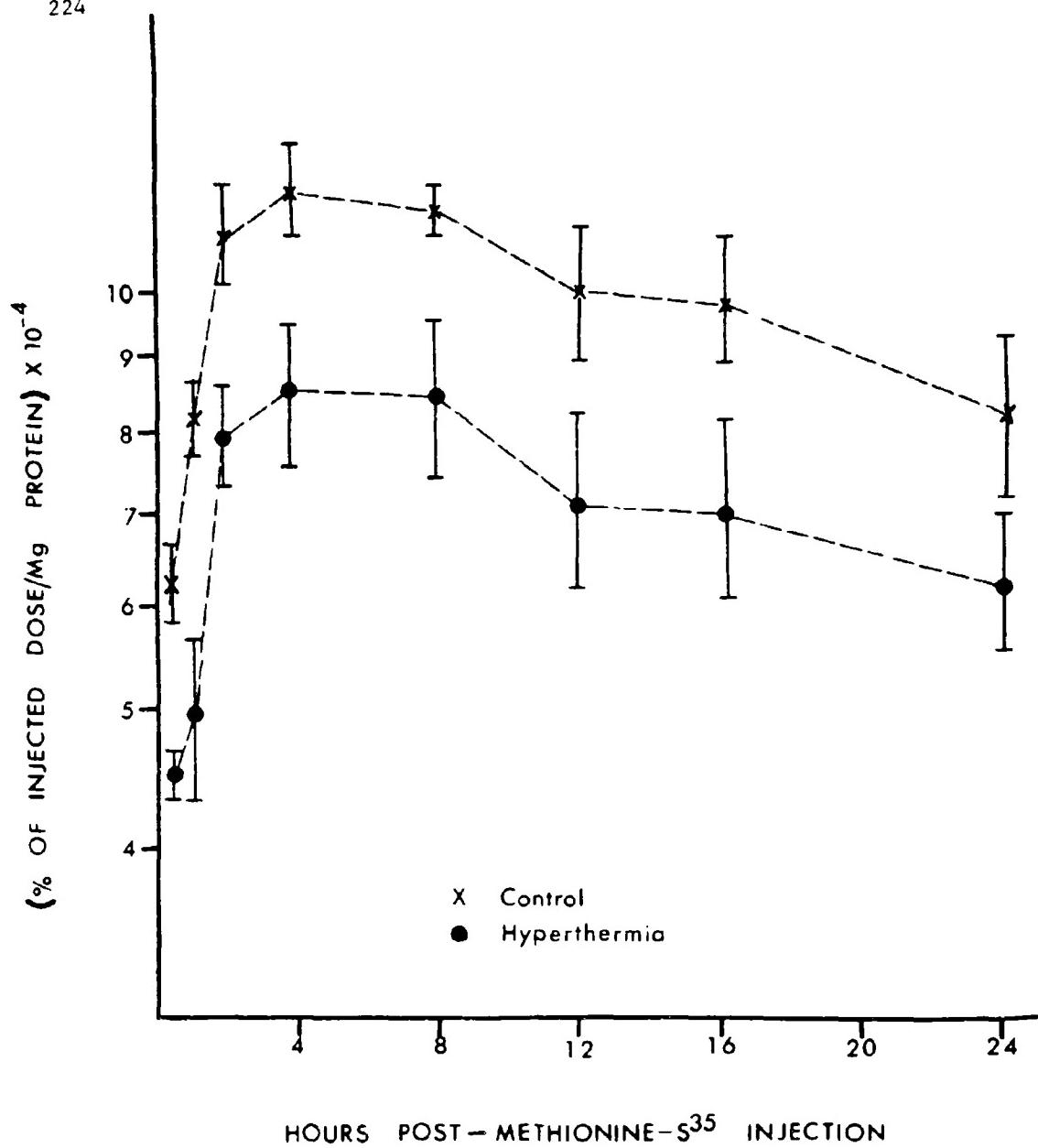


FIGURE 1. EFFECT OF HYPERTERMIA ON IN VIVO INCORPORATION OF 1-METHIONINE-S³⁵ INTO RABBIT SERUM PROTEIN (MEAN \pm 1 SE).

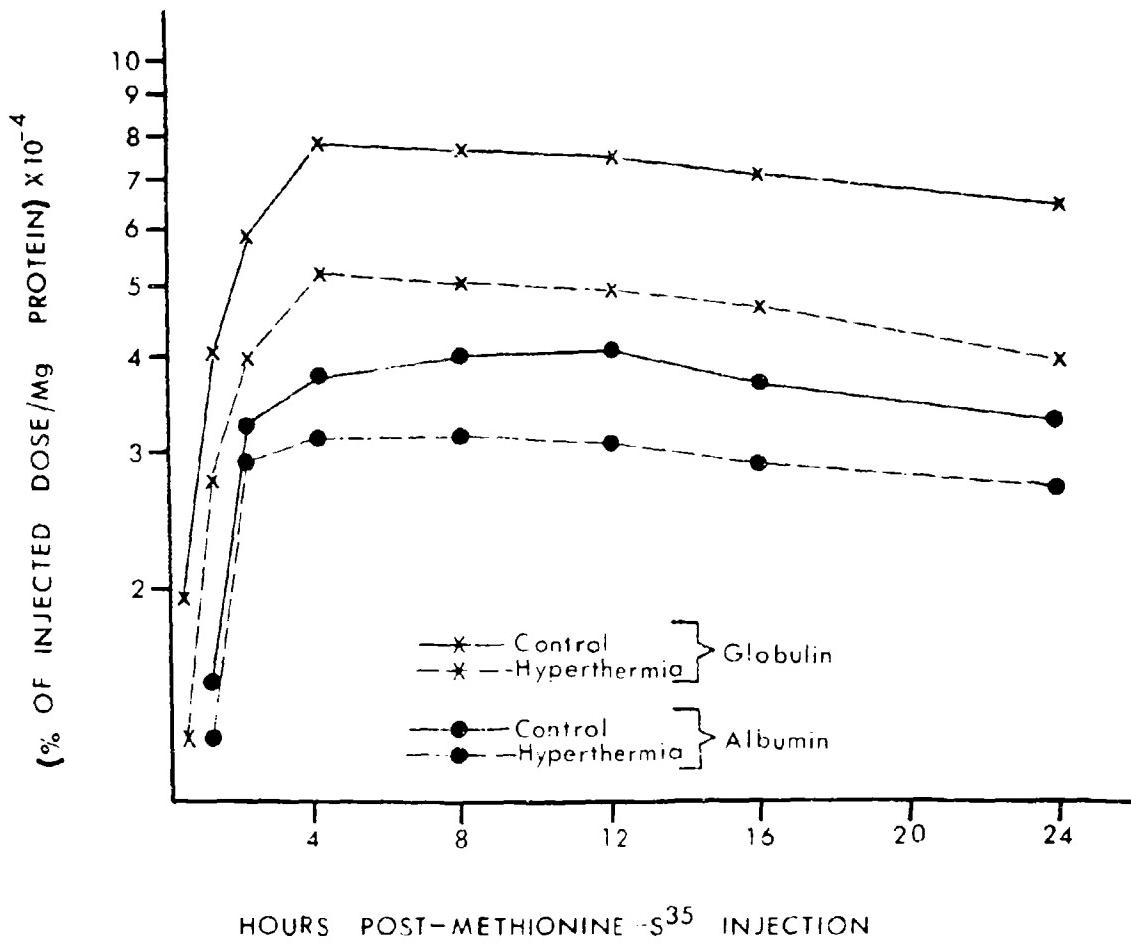


FIGURE 2. INCORPORATION OF S^{35} RADIOACTIVITY INTO SERUM ALBUMIN AND GLOBULIN OF HYPERTHERMIC AND CONTROL RABBITS.

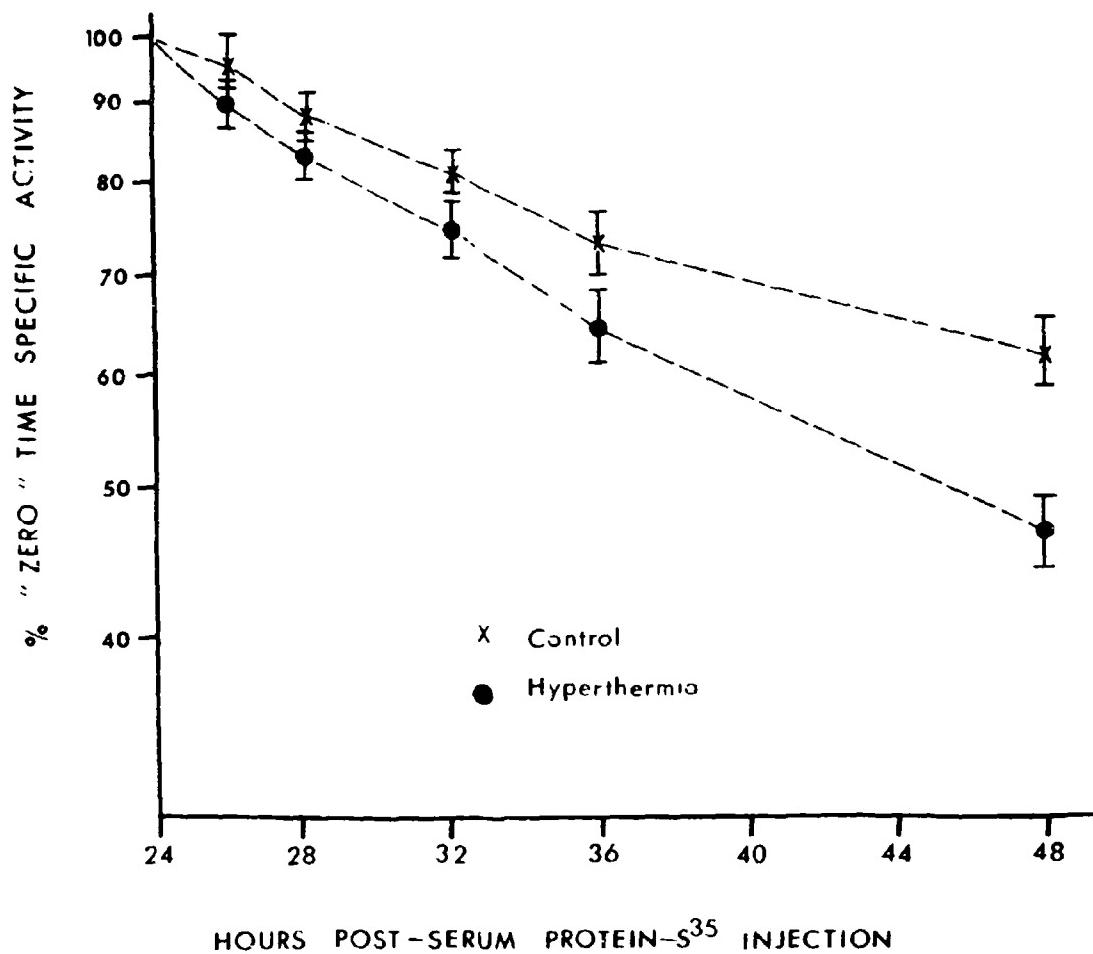


FIGURE 3. EFFECT OF IN VIVO HYPERTERMIA ON THE DEGRADATION RATE OF INJECTED S^{35} LABELED SERUM PROTEIN (MEAN \pm 1 SE).

TABLE I. EFFECT OF IN VIVO HYPERTERMIA ON PROTEIN METABOLISM IN VIVO IN RABBITS

	CONTROL		HYPERTERMIA		P
	No.	Mean ± SE	No.	Mean ± SE	
Synthesis % injected ^{a/}	14	0.00180 ± 0.00092	11	0.00845 ± 0.00069	< 0.05
Catabolism % of O time ^{b/}	12	37 ± 2.9	12	52.5 ± 3.8	< 0.01

a. % injected dose/mg protein at 4 hr.

b. % of "C" time activity at 24 hr.

Liver Brei Studies.--The problems inherent in turnover studies in an mammalian system are known. To help explain our observations of changed protein metabolic activity, in vitro preparations of liver are utilized. A liver brei suspension was made from animals treated exactly as had been the animals in the in vivo studies. The rate of incorporation of Me-S³⁵ into protein by liver brei from hyperthermic animals is only about $\frac{1}{2}$ that of the controls (0.000131 vs. 0.000251% respectively). This supports the hypothesis that hyperthermia has affected protein synthesis by the liver.

Homologous S³⁵-labeled rabbit serum was incubated with liver brei to follow catabolism. Hyperthermic liver incubated at the same temperature as the control liver (37.5 C) degraded about twice as much protein as the control during the same time interval (20.6 vs 10.7 respectively). This also supports our in vivo findings. Table II summarizes the

TABLE II. EFFECT OF IN VIVO HYPERTERMIA ON PROTEIN METABOLISM IN VITRO IN LIVER BREI

	CONTROL		HYPERTERMIA		P
	No.	Mean ± SE	No.	Mean ± SE	
Synthesis % added ^{a/}	10	0.000251 ± 0.000024	11	0.000131 ± 0.000015	< 0.01
Catabolism % loss ^{b/}	10	10.7 ± 1.3	10	20.6 ± 2.5	< 0.01

a. % of Me-S³⁵ added to incubation mixture incorporated/mg protein.

b. % of loss of protein radioactivity during incubation period/hr.

effect of in vivo hyperthermia on protein metabolism by liver brei. It is seen that a depression of Me-S³⁵ incorporation, as well as an increase in S³⁵-serum degradation, has occurred in the liver brei from hyperthermic animals.

From our findings in vivo and the supporting in vitro data, we can conclude that hyperthermia causes a depression of protein synthesis. This may come about as a result of damage to the enzyme system. Furthermore, it could be expected that the catabolic rate would be increased with increased temperature in the usual way. The fact that the catabolic rate continues at an increased rate when the tissue has been removed and incubated at control temperature, must mean that some additional mechanism is also operative. Although the hyperthermic conditions in the animal could have affected pH, inhibitors, or activated inactive enzymes which might not have been corrected by in vitro incubation, we would propose that this continued effect at control temperatures might result from a more fundamental process. In the light of the decreased protein synthesis during hyperthermia, the protein needs of the animal were not being met. In order to simulate protein synthesis, endogenous protein was being broken down to produce amino acid precursors. However, these precursors could not be utilized and the feedback cycle was continued. This new state of metabolism was carried out even in vitro in order to meet the tissue demand. Thus, we can conclude that hyperthermia affects protein turnover in 2 ways: (1) by depressing protein synthesis and (2) by temperature feedback stimulation of catabolic activity.

Lysosomal Cathepsin Studies.--In view of the observed increased rate of catabolism in the hyperthermic state as reflected by the in vivo and in vitro catabolism studies, it seemed reasonable to assume that the cathepsins, intracellular proteolytic enzymes,^{24/} might play a fundamental role. de Duve et al.^{25/} proposed the concept that cellular hydrolases are contained in an intracytoplasmic organelle which they named lysosome. Cathepsins were demonstrated in lysosomes.^{26/} Therefore, in order to study some of the properties of these enzymes, as related to our studies, we initiated steps to isolate lysosomes from liver obtained from control and experimental animals. From Table III it is seen that the pH optimum for both control and hyperthermic isolated fractions was the same, pH 5.0. Cathepsins have been shown to require -SH activating compounds.^{23,24/} Cysteine was used as an activator and the results are shown in Table IV. It is seen that both groups show a 4-fold increase in activity in the presence of cysteine.

The effect of in vitro temperature on the lysosomal cathepsins isolated from control and hyperthermic liver was next investigated. These results are shown in Table V. These data show that the enzymes from hyperthermic liver are significantly more active at both incubation temperatures (37, 42 C) than the control liver enzymes. These results are in agreement with our findings in the brei experiments (Table II). Moreover, if the activity of control and hyperthermic preparations is compared against itself at the 2 incubation temperatures, it is seen that, at the higher incubation temperature there has been stimulation of activity.

TABLE III. EFFECT OF pH ON THE PROTEOLYTIC ACTIVITY OF LYSOSOMAL CATHEPSINS FROM LIVER

EXPERIMENT NO.	NET CPM/MG LYSOSOMAL PROTEIN/30 MIN							
	Control				Hyperthermia			
	3.8 ^{a/}	5.0	6.0	7.0	3.8	5.0	6.0	7.0
1	478	543	350	168	536	904	368	277
2	358	475	282	203	180	484	128	104
3	283	385	140	127	792	1227	444	356
4	375	578	245	193	308	496	296	192
TOTAL	1494	1981	1017	691	1816	3111	1236	929
MEAN	374	495	254	173	454	777	309	232

a. pH of incubation mixture.

TABLE IV. EFFECT OF CYSTEINE ON THE PROTEOLYTIC ACTIVITY OF LYSOSOMAL CATHEPSINS

EXPERIMENT NO.	NET CPM/MG LYSOSOMAL PROTEIN/30 MIN			
	Control		Hyperthermia	
	With -SH	Without -SH	With -SH	Without -SH
1	340	164	252	115
2	442	56	796	194
3	482	60	581	104
4	70	15	250	30
5	144	44	357	119
TOTAL	1478	339	2236	562
MEAN	296	68	447	141

TABLE V. EFFECT OF INCUBATION TEMPERATURE ON PROTEOLYTIC ACTIVITY OF LYSOSOMAL CATHEPSINS ISOLATED FROM RABBITS

EXPERIMENT NO.	CONTROL				HYPERTHERMIA			
	37 C		42 C		37 C		42 C	
	%/mg ^a /	μg/mg ^b /	%/mg	μg/mg	%/mg	μg/mg	%/mg	μg/mg
1	1.76	118	1.84	124	4.62	171	6.05	224
2	1.07	67	1.80	117	4.52	336	4.53	343
3	1.46	41	1.70	65	5.06	378	5.16	394
4	0.74	57	0.78	67	0.60	24	0.92	37
5	0.37	30	0.37	29	1.76	78	2.34	106
6	0.62	26	1.47	60	6.31	266	7.09	295
7	3.40	194	5.54	316	5.92	252	8.47	360
8	0.76	36	1.00	55	5.90	235	7.50	298
9	3.74	131	3.88	136	--	--	--	--
TOTAL	13.92	700	18.38	969	34.69	1740	42.06	2057
MEAN	1.54	77.8	2.04	107.7	4.33	216.3	5.25	257.2
SE	±1.23	±56.0	±1.64	±86.0	±2.34	±123	±2.60	±126

a. Net % incubated S³⁵-labeled protein radioactivity released into the TCA soluble fraction/mg lysosomal protein/30 min.

b. μg S³⁵-labeled protein degraded/mg lysosomal protein/30 min.

To determine other properties of the cathepsins from control and experimental livers, kinetic data were obtained. Excess enzyme was incubated with varying amounts of S³⁵-labeled protein as substrate. Table VI shows the results of these experiments. The amount of protein degraded has been used as an indicator of velocity of the reaction. A form of the Lineweaver-Burk interpretation of the data was undertaken. The best straight line for each group was obtained by the "least squares" method.²⁷

TABLE VI. EFFECT OF HYPERTERMIA ON THE KINETICS OF ISOLATED LYSOSOMAL CATHEPSINS

ENZYME GROUP	TEMPERATURE °C	EXPERIMENT NO.	SUBSTRATE CONC./VELOCITY (Km/V)			
			3000 µg ^a /	1300 µg	650 µg	360 µg
CONTROL	37	1	161.0	83.2	67.0	46.0
		2	29.4	14.3	13.3	12.2
		3	132.0	74.5	31.8	24.3
		4	26.8	16.9	13.5	9.2
		5	162.0	79.4	65.0	45.0
		6	162.0	106.0	43.0	40.0
		7	25.8	13.9	9.6	7.5
HYPER-THERMIA	42	Total	699.0	388.2	243.2	184.2
		Mean	99.0	55.5	37.7	26.3
		1	78.0	56.0	39.0	31.0
		2	18.0	11.6	10.6	9.6
		3	100.0	55.0	39.0	32.0
		4	25.8	16.6	12.5	11.5
		Total	331.8	139.2	101.1	84.1
HYPER-THERMIA	37	Mean	55.5	34.8	25.3	21.0
		1	110.0	85.0	36.0	20.0
		2	127.0	71.1	57.0	31.0
		3	39.0	19.0	11.0	7.0
		4	57.0	39.0	34.0	32.0
		5	16.0	8.0	7.0	6.0
		6	17.0	11.0	7.0	6.0
HYPER-THERMIA	42	Total	366.0	233.0	152.0	102.0
		Mean	61.0	38.8	25.5	17.0
		1	111.0	85.0	36.0	20.0
		2	53.0	35.0	23.0	18.0
		3	14.2	6.7	4.1	3.0
		4	13.3	8.1	6.2	4.6
		5	57.5	48.5	30.0	29.0
		6	11.8	6.4	5.5	4.5
		Total	260.8	189.7	104.8	79.1
		Mean	43.5	31.5	17.5	13.2

a. µg S³⁵-labeled protein incubated.

For the preparations from control rabbits, an increase in incubation temperature caused an increase in velocity at the higher incubation temperature. Since the intercept for these 2 curves remained the same, and since this intercept represented Michaelis-Menten constant/maximum velocity (Km/V), a decrease in the constant must occur for this relationship to remain the same. Therefore, temperature has caused an increase in the turnover number of these enzymes.

The results for the experimental rabbits indicate that at 37 C, the velocity for this group is higher than that for the control group at 37 C; in fact, its velocity approaches that of the control fractions incubated at 42 C. Furthermore, from the curves of the experimental rabbit enzymes at the 2 temperatures, it was seen that the velocity and Km remained essentially the same.

It appears then, that hyperthermia causes an increase in catabolic activity of the cathepsins either by promoting the production of activators or destruction of inhibitors of these enzymes. This effect can be produced by in vivo or in vitro temperature stimulation. Furthermore, since the kinetic activity of cathepsins from hyperthermic animals remains essentially the same even when incubated at the lower temperature, the temperature effect on this system appears to be irreversible. In fever with infection, a negative N balance has been noted to persist into convalescence, even when the fever was absent.^{3,67} It may be possible that the presence of an increased number of activated, formerly inactive, enzymes, and/or alteration of the reactive sites might contribute to this observation. This increased activity would be expected to persist until the irreversibly changed molecules are destroyed and replaced by normal molecules.

SUMMARY

A study of the effect of hyperthermia on protein metabolism, using in vivo and in vitro techniques has revealed the following:

Hyperthermia, in vivo, was seen to cause about a 40% depression of protein synthesis. In vitro studies using liver brei supported this. It was observed that incorporation of Me-S³⁵ into liver protein of the experimental brei was about one-half that of controls.

In vivo studies indicate that the catabolic rate of hyperthermic animals is about 30% faster than controls. In vitro studies with liver brei indicate that the catabolic activity of experimental hyperthermic brei is higher than control brei.

The increased catabolic activity was attributed to increased catheptic activity. This greater activity was demonstrated at both 37 and 42 C.

Kinetic data indicate that increased temperature in vivo or in vitro is responsible for an irreversibly increased catheptic activity.

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TRACE ELEMENTS

Captain David G. Van Ormer, MSC*

In considering the variety of possible changes in host metabolic systems that might occur during infectious illness, it seemed possible that leads could be obtained through the study of trace elements.

Although little is known concerning the role of input trace metals in biologic systems, several metals have been shown to function as important cofactors for enzymes, to form complexes with proteins, or occasionally, to produce disease if present in excess or inadequate amounts. Although mineral metabolism has been systemically reviewed,^{1,2} virtually nothing is known about trace metal metabolism during acute infectious illness.

The technique of atomic absorption spectrophotometry was adopted in the Medical Unit to initiate such studies. Zinc was selected for analysis because it is normally present in human urine in concentrations detectable by our instruments without the need for prior concentration or extraction. This work was initiated by Dr. Beisel and Miss Karen Bostian. It was first determined that urine could be collected and stored frozen in disposable plastic containers without changing the concentration of Zn.

Urine specimens were collected during consecutive 24-hr periods throughout a number of studies involving the exposure of volunteers to infectious microorganisms.

Figure 1 depicts our most complete study, one involving 8 men exposed to Pasteurella tularensis. The fever index is shown at the top. Shown as a horizontal stippled band is the mean \pm 1 SE of all control-period collections. There was a very small day-to-day variability of the group average during the preexposure control period. Following challenge, there occurred an initial fall in Zn excretion in conjunction with the onset of symptoms and fever. This drop was followed by a decided and rather protracted increase. Of the various elements studied in earlier metabolic balance investigations, the pattern seen here resembled closely that observed with urinary phosphorus.

Figure 2 shows the results obtained for 3 men infected with Q fever. Little change in urinary Zn was noted during most of the long incubation period. There then occurred a presymptomatic fall again followed by a postfebrile period.

Data from 8 subjects during sandfly fever infection are shown in Figure 3. Note the significant fall in urinary Zn excretion at the

* U. S. Army Medical Unit.

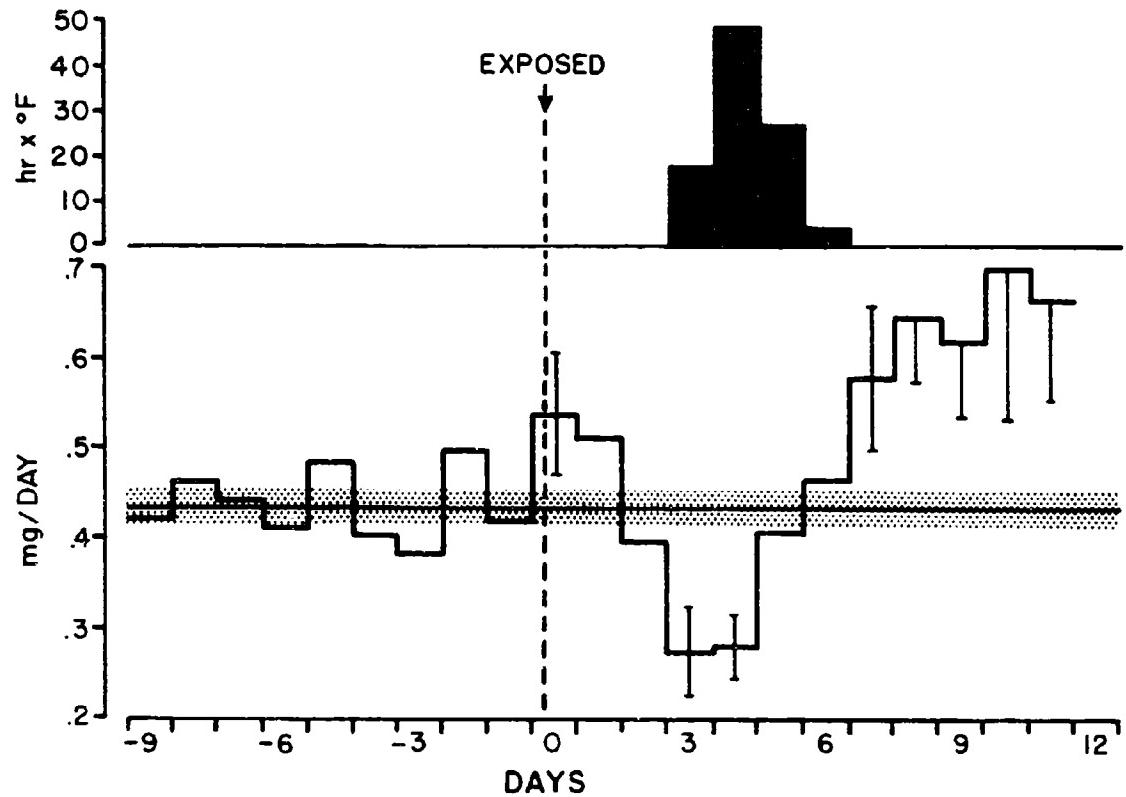


FIGURE I. URINARY ZINC EXCRETION IN TULAREMIA
(AVERAGE OF 8 SUBJECTS).

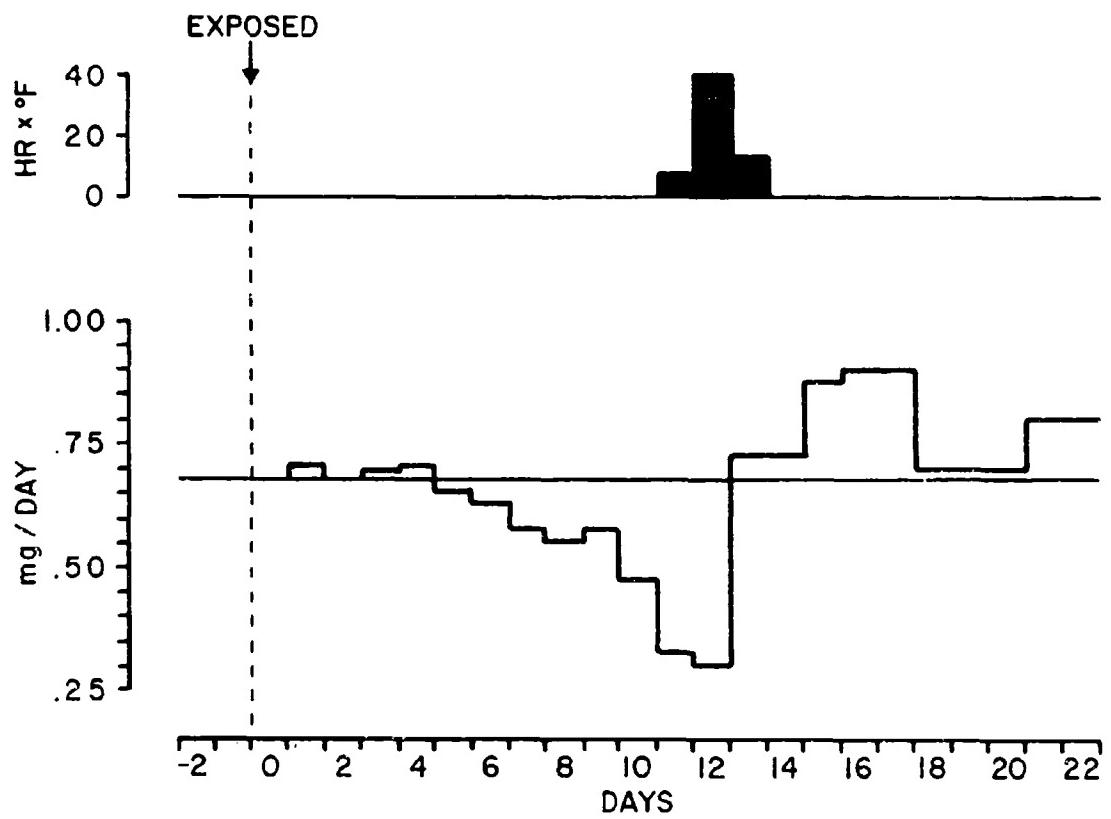
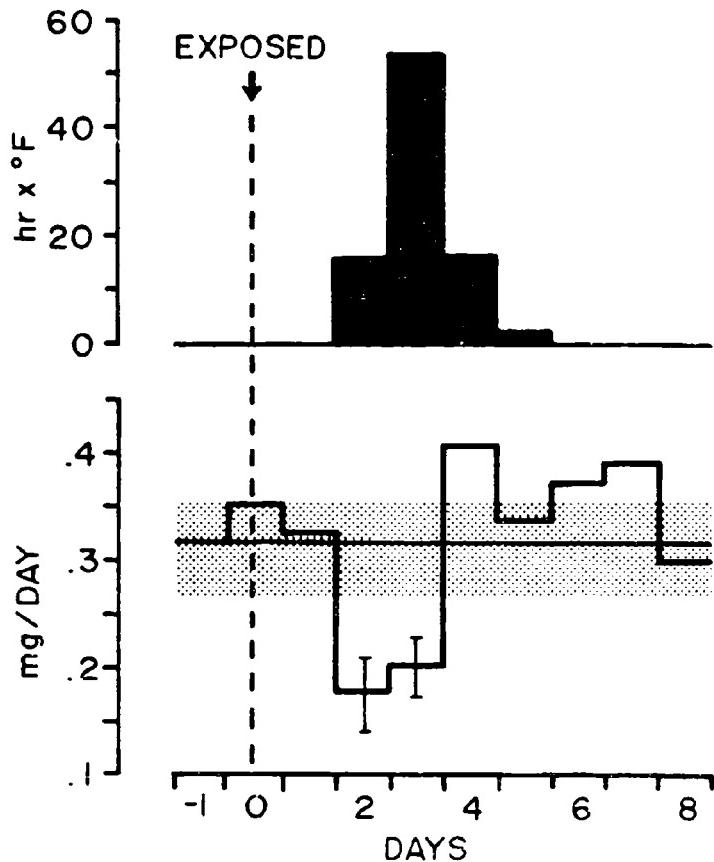


FIGURE 2. URINARY ZINC EXCRETION IN Q FEVER
(AVERAGE OF 3 SUBJECTS).



**FIGURE 3. URINARY ZINC EXCRETION
IN SANDFLY FEVER
(AVERAGE OF 8 SUBJECTS).**

onset of clinical illness. In none of the studies did sham-exposed control subjects show appreciable changes.

Data from 6 subjects inoculated with 17-D strain yellow fever vaccine are plotted in Figure 4. They remained entirely asymptomatic. A small fall can be seen on day 6.

Another group of 8 men inoculated with a living, attenuated plague vaccine failed to show a detectable change in Zn excretion despite the consistent occurrence of a local skin response together with an occasional local lymph node reaction and slight fever. Thus, changes in excretion of this trace metal seemed greatest in association with clinical illness produced by generalized systemic infections. The meaning, mechanism, or ultimate significance of these responses must await further study.

Other trace elements will undoubtedly offer greater difficulties in methodology. The detection limits of atomic absorption are apparently not low enough to permit direct detection of many trace metals in normal urine. Preconcentration is possible by either solvent extraction^{3/} or ion exchange.^{4/} The former method provides greater effective concentration because aspiration from an ester or ketone solution provides a 3- or 4-fold increase in aspiration efficiency of the flame over aspiration from aqueous solution. Our own experience along with that of other investigators,^{5,6/} demonstrates that many trace metals cannot be extracted directly from urine with acceptable precision. Digestion procedures for urine are tedious and are still in need of rigorous investigation. The best digestion reagent employed has been a mixture of concentrated HNO₃ and H₂SO₄ and 70% HClO₄ (3:1:1). For several trace metal ions, extraction from urine digests into methyl iso-butyl ketone is practicable as the ammonium pyrrolidine dithiocarbamate chelate. This system, however, frequently extracts with good precision only from concentrated electrolyte solutions and only when the sample is heated to incipient boiling after addition of the chelating agent and before extraction. We have achieved good precision by extraction from a 25% KCl solution. The feasibilities of other chelating systems are being considered and attempts for at least partial automation of the procedure are still under investigation.

SUMMARY

Recent improvements in methodology make it possible to detect and quantitate many of the trace elements in biological fluids despite their low concentrations. Urinary zinc was selected for initial study because it could be detected by atomic absorption spectroscopy without the need for prior concentration or extraction. Following challenge of normal volunteers with P. tularensis, an early fall in urinary Zn excretion accompanied the onset of symptoms and fever; this early drop was followed by a marked and prolonged increase. Similarly, in subjects exposed to Q fever, little change in urinary Zn occurred in the early portions of the long incubation period; then it showed a presymptomatic fall followed by a postfebrile rise. In neither study did sham-exposed control subjects show such a change.

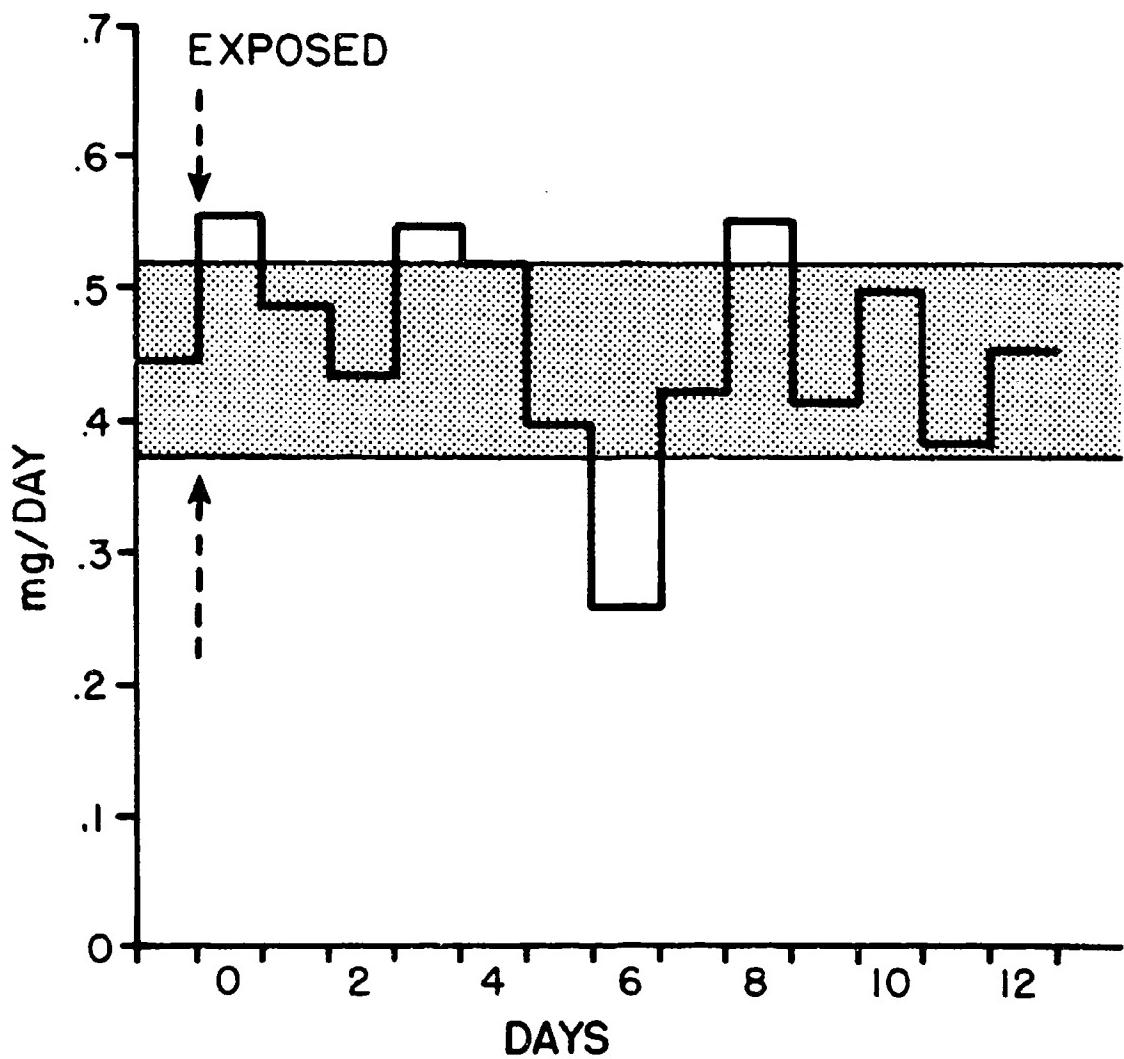


FIGURE 4. URINARY ZINC EXCRETION
AFTER 17-D YELLOW
FEVER VACCINE
(AVERAGE OF 6 SUBJECTS).

To study other trace elements, several methods will be required to increase their concentration prior to analysis. Preconcentration, digestion procedures, chelation, and solvent extraction are currently under investigation to provide for a future method that will be precise, rapid, consistent, and relatively simple.

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DISCUSSION

DR. YOUNG: I wonder if the changes you observed in Zn excretion reflect possible changes in the activity of the mineralized tissues of the body, since trace minerals are found in relatively high concentrations in the bony tissue.

DR. VAN ORMER: It is possible that increased Zn excretion reflects changes in bone mineral structure. At this point we are really in the analytical and descriptive phases of our work, and the mechanisms behind these changes must wait until we are on a little firmer analytical basis and can repeat these experiments for other metals.

SERUM GLYCOPROTEIN CHANGES IN INFECTIOUS DISEASES

Captain Albert S. Klainer, MC*

Changes in serum proteins following infection have been studied for some time. Early impetus for this was the hope that patterns diagnostic for specific infectious diseases would emerge. What did result, however, was a constellation of changes which probably reflected nonspecific response to infection or inflammation. Acute infection is characterized by an increase in α_1 -globulins and a decrease in albumin; chronic infection, by a decrease in albumin and an increase in γ -globulin. The majority of studies in the past, however, have been done in patients in whom infection was established. Dr. Gray¹⁷ was one of the first to study prospectively serum proteins in acute infection. During acute controlled respiratory-acquired tularemia he demonstrated a decrease in serum albumin and an increase in α_2 -globulin 6-7 days after infection, or 1-2 days after the onset of fever. He demonstrated more pronounced changes in serum glycoproteins; an earlier and more significant decrease in serum glycoalbumin and an elevation of α_1 -glycoprotein; both changes were demonstrable 1-2 days before the onset of fever. Because of our interest in studying the metabolism of infection especially during the incubation period, Dr. Gray's studies suggested that investigation of infection-induced serum glycoprotein changes might have great potential. In addition, there has been some evidence that glycoproteins may respond with some specificity to diverse pathologic stimuli.

Glycoproteins are characterized by having carbohydrate firmly linked to a peptide moiety by covalent bonds; their sugar constituents are the amino sugars, glucosamine and galactosamine; the neutral sugars mannose, galactose, fucose and in a few cases glucose; and various derivatives of neuraminic acid collectively referred to as sialic acid.

Examples of glycoproteins important in human metabolism are: ceruloplasmin, haptoglobin, transferrin, prothrombin, fibrinogen, follicle-stimulating hormone, interstitial cell-stimulating hormone, chorionic gonadotropin, erythropoietin, thyroid-stimulating hormone, and thyroglobulin. In addition there are α_1 -, α_2 -, β -, and γ -glycoglobulins which can be identified by electrophoretic separation; only a small percentage of albumin contains bound carbohydrate. The 19S γ -globulin contains about 10% carbohydrate, 7S, about 3%. The carbohydrate moiety is characteristic for many glycoproteins, but for the purpose of this discussion I will be concerned only with gross infection-induced changes in serum glycoglobulins.

Before describing infection-induced changes, let me refamiliarize you with the electrophoretic separation and densitometric quantitation of serum glycoproteins. We have adapted a rapid method for this determination

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on cleared cellulose acetate strips which has eliminated the need for concentration of biologic specimens and the problem of fading.^{2/}

Figure 1 shows electrophoretic patterns for normal serum glycoproteins and proteins and the percentages by each method. The predominant band on the left is α_2 -glycoglobulin; on the right, albumin, as conventionally seen.

The results of preliminary studies of the effect of carefully controlled infection on serum glycoproteins in animals and humans which follows represent a further insight into the early pathophysiology of infection. They have the added advantage of prospective studies which with the exception of Dr. Gray's^{1/} work is lacking from the myriad of information previously gathered about this group of serum proteins.

Figure 2 represents changes in serum glycoglobulins during the first 24 hr of experimentally-induced pneumococcal infection in CD-1 strain male mice. Each animal was challenged subcutaneously on the back with 2×10^6 virulent pneumococci. Controls received sterile tryptose phosphate broth. The horizontal bands represent the mean \pm 1 SE of control values for each fraction. The diamonds represent values significant at $p < 0.001$. Glycoalbumin is not depicted on this or the following figures because of its small content of bound carbohydrate and because it remains relatively stable. It has been included, however, in the calculations. Note that there is a striking and significant decrease in α_1 -glycoglobulin starting at 12 hr and continuing until termination of the study at 24 hr. These changes are accompanied by a rise in α_2 - and β -glycoglobulins, whereas γ -glycoglobulin remains stable. Glycoglobulins are reported in per cent; we feel that this is valid at this early point in our experiments because the literature suggests that the absolute amount of total serum glycoproteins does not change significantly this early in infection or inflammation. We are in the process of obtaining more quantitative results to document this.

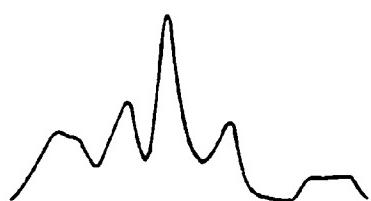
Figure 3 depicts on the left densitometer curves for individual mice 4, 16 and 20 hr after subcutaneous infection with pneumococci. Note the disappearance of the α_1 peak. Death in this group of CD-1 strain male mice does not occur for 36-48 hr so that these changes cannot be described as terminal. In addition rats injected subcutaneously with only 100 pneumococci showed strikingly similar changes.

The effect of respiratory-acquired Pasteurella tularensis infection upon serum glycoproteins was studied. Two groups of subjects were exposed to 25,000 viable SCHU-S4 cells via aerosol. The only difference in the 2 groups was that the first received tetracycline therapy; the second, streptomycin.

Figure 4 shows serum glycoglobulin levels prior to, during, and following infection. The breaks in the curve represent days on which no samples were obtained. The heavy dots represent values significant at $p < 0.01$;

SERUM GLYCOPROTEINS

2-8% - Alb. - 45-55%
10-16% - α_1 glob - 5-8%
27-36% - α_2 glob - 8-13%
20-27% - β glob - 11-17%
20-28% - γ glob - 15-25%



SERUM PROTEINS

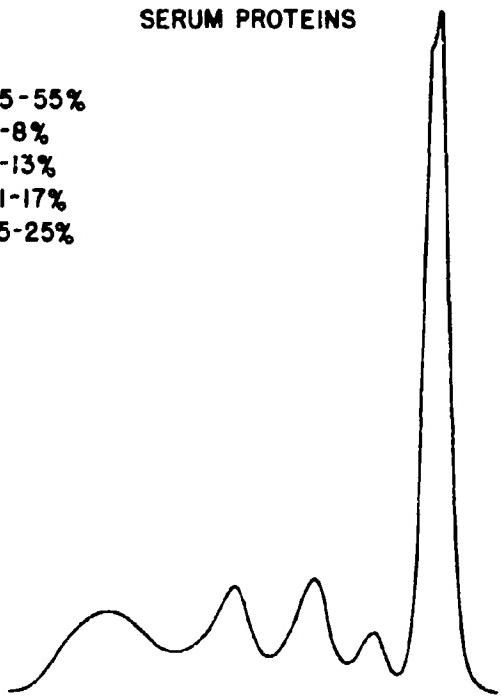


FIGURE I. ELECTROPHORETIC PATTERNS OF NORMAL HUMAN SERUM PROTEINS AND GLYCOPROTEINS.

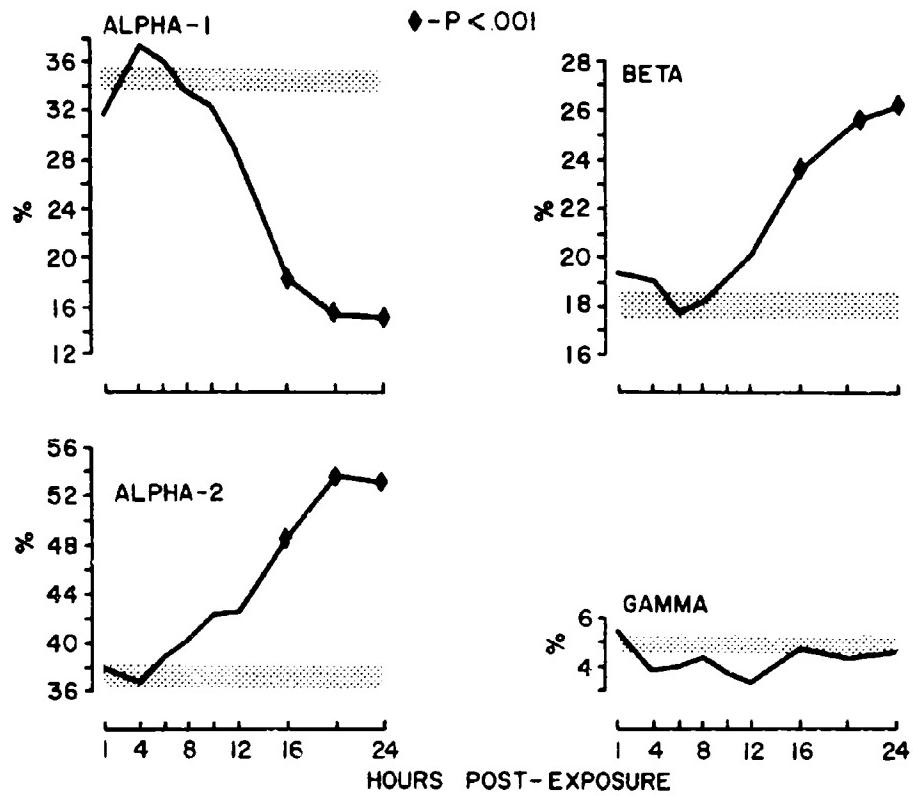


FIGURE 2. GLYCOPROTEIN CHANGES IN PNEUMOCOCCAL INFECTION IN MICE.

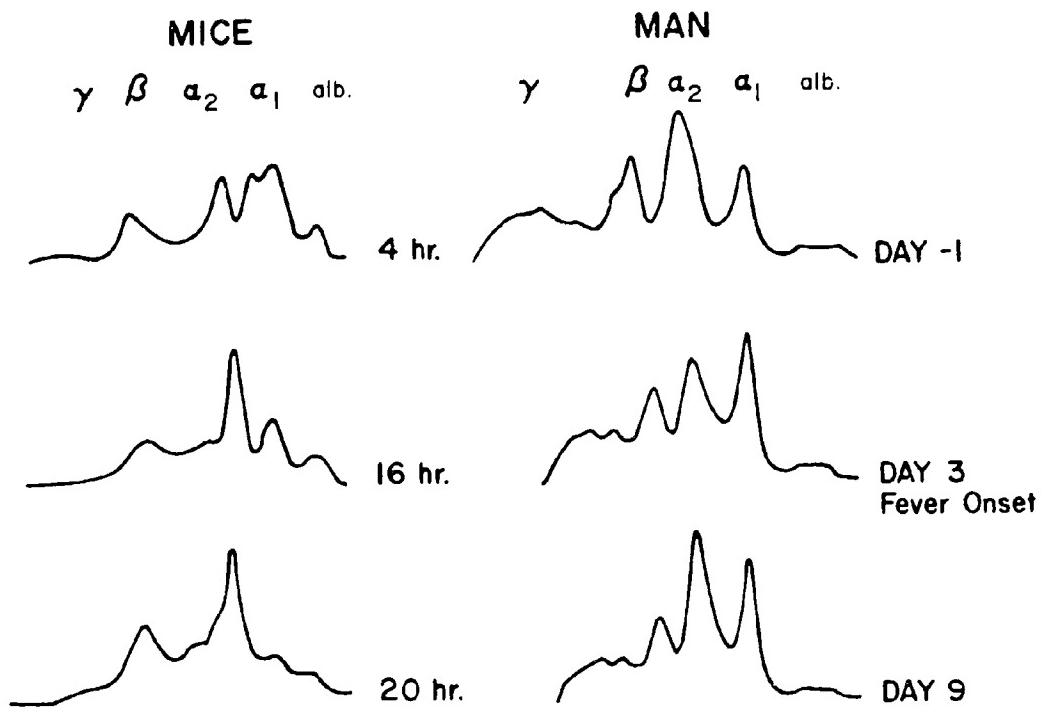


FIGURE 3. SERUM GLYCOPROTEIN ELECTROPHORETIC PATTERNS. LEFT: MOUSE PNEUMONIA . RIGHT: HUMAN RESPIRATORY TULAREMIA .

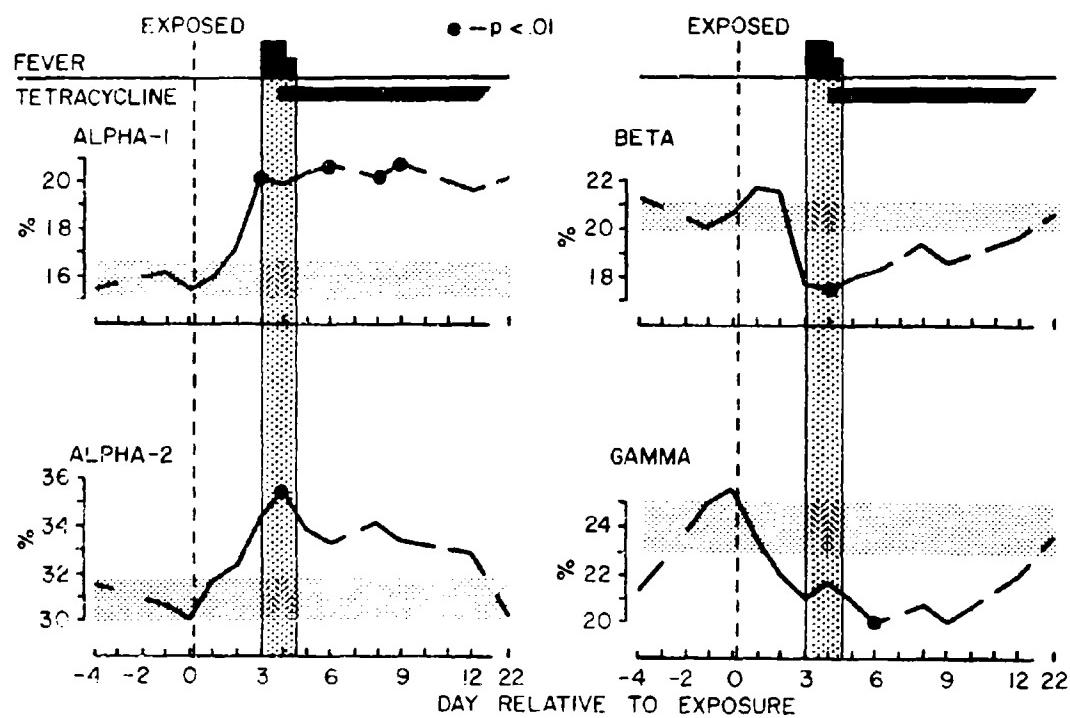


FIGURE 4. GLYCOPROTEIN CHANGES IN ACUTE RESPIRATORY TULAREMIA.

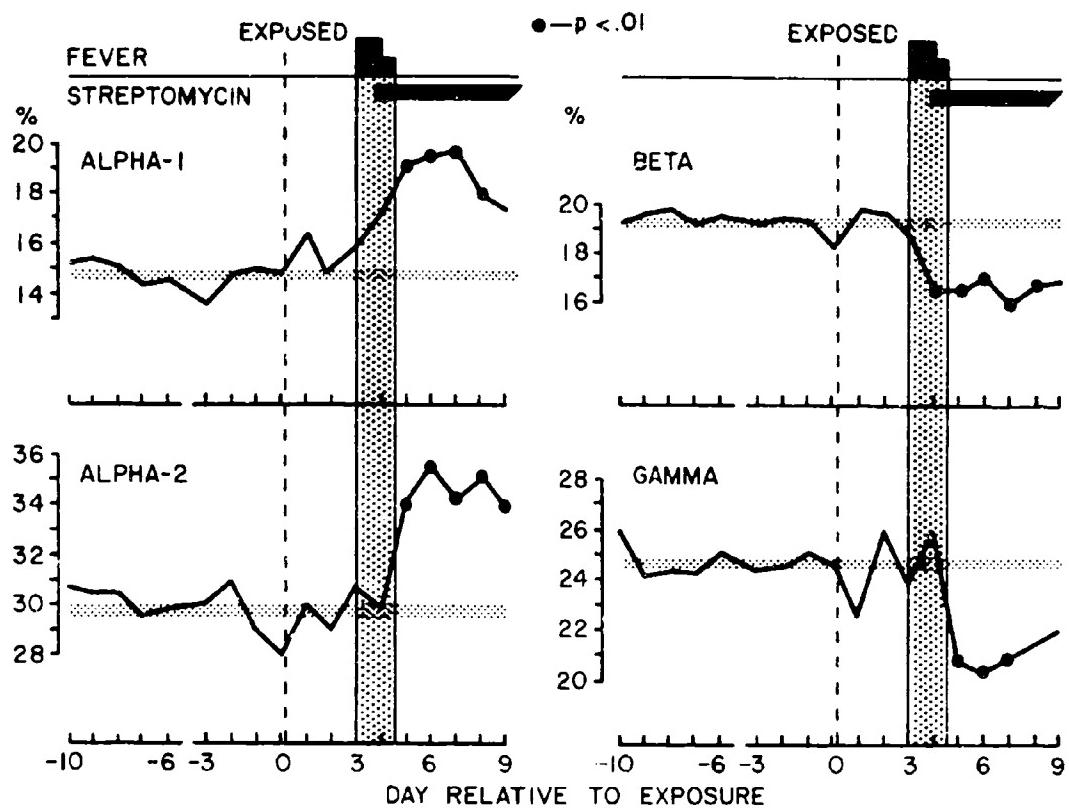


FIGURE 5. GLYCOPROTEIN CHANGES IN ACUTE RESPIRATORY TULAREMIA.

many neighboring points on the curve are significant at the 2 or 5% level but are not designated as such for the sake of clarity. The horizontal dotted band represent the mean \pm 1 SE of all preinfection samples for each fraction. The dashed vertical line represents the day of infection, day 0; the vertical bar, the period of fever; and the solid horizontal bar the period of tetracycline therapy. Note the significant rise in α_1 -glyco-globulin which just precedes the onset of fever and persists for almost 2 weeks postinfection. An initial rise in α_2 -glycoglobulin during the febrile period is seen, as are concomitant decreases in β - and γ -glyco-globulins; the changes in the α_2 -, β -, and γ -fractions returned to normal values within 2 weeks. Controls also receiving antibiotics did not show similar changes suggesting that the patterns observed are infection-induced and not attributable to therapy.

Figure 5 shows the results of respiratory-acquired tularemia in a second group of subjects. You will note that the pattern of changes is the same as for the first group. The significant glycoprotein changes, however accompany and follow, rather than precede, clinical illness. In this study serum was obtained every day before, during, and after infection. The second study substantiates the fact that the changes observed are reproducible and significant.

Referring to the densitometer curves in Figure 3 on the right, the most significant gross change for man infected with tularemia is the increased α_1 band which, on the day of onset of fever, day 3 postinfection, is obviously increased. Following infection the α_1 band remains elevated but does not display the same degree of prominence.

To determine the effect of a virus infection on serum glycoproteins, sandfly fever infection of 8 volunteers was studied. The virus, in 1 ml of filtered human plasma, was injected intravenously. Seven of the 8 developed typical illness; one subject developed only low-grade fever which lasted about 36 hr. Figure 6 shows daily serum glycoprotein levels for 3 days prior to infection, the day of infection, and for 8 days following infection. Day 0 is the day of exposure. The vertical band represents the 3 days of fever in infected subjects. Typical illness is characterized by an incubation period of 2 days and 48 hr of fever; the disease is self-limited, but characterized by a variable period of post-febrile malaise and asthenia.

Although greater man-to-man variability was observed with this group than previously, a characteristic pattern emerged. There was a significant elevation in α_1 -glycoglobulin and a significant decrease in γ -glycoglobulin just after the febrile period. No significant changes were observed in either α_2 - or β -glycoglobulins. No changes in serum glycoproteins occurred in control subjects, and there was no obvious relationship between glycoprotein changes and the severity of illness. We are presently repeating this study in a second group of volunteers and have substantiated the results just described.

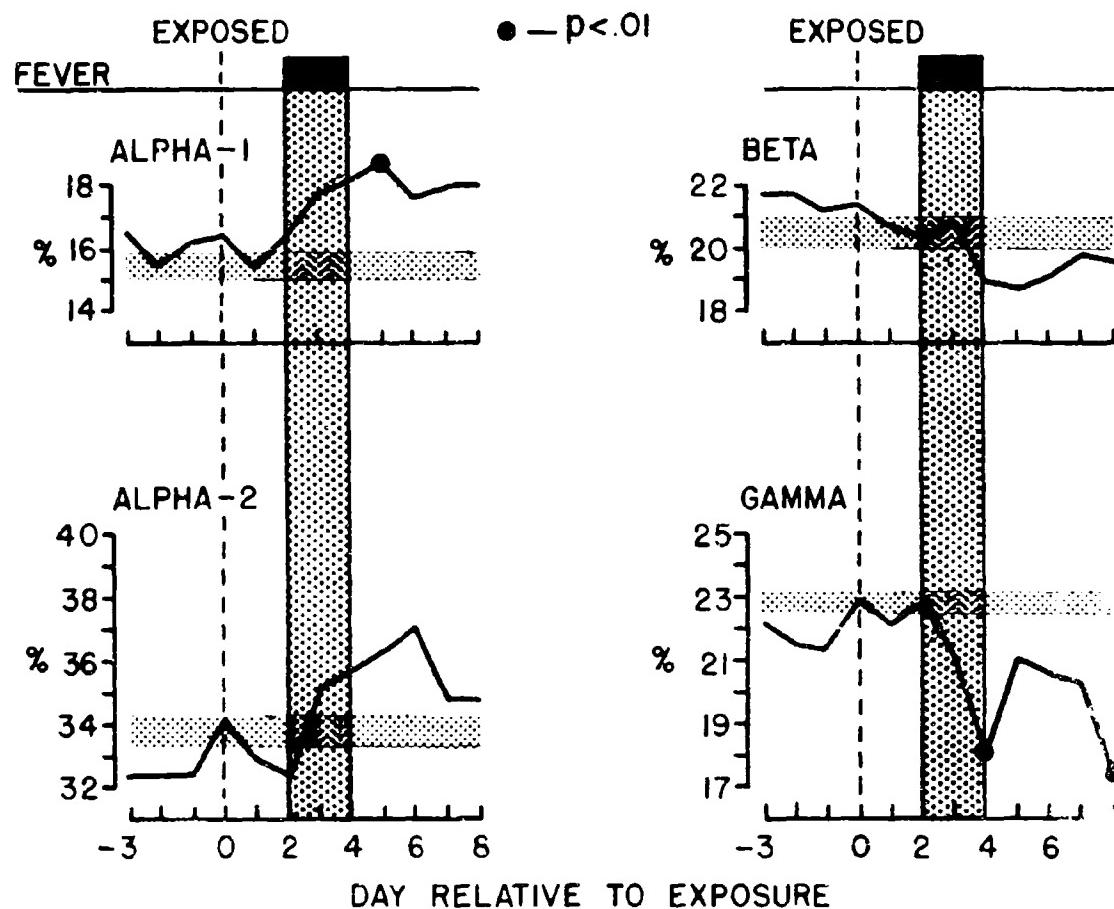


FIGURE 6. GLYCOPROTEIN CHANGES IN SANDFLY FEVER.

Although the studies reported are preliminary and simple, a number of thought-provoking observations have emerged which have stimulated plans for future study.

Table I summarizes the significant differences between each of the studies described. Within 12-16 hr following pneumococcal infection in

TABLE I. SUMMARY OF GLYCOPROTEIN CHANGES FOLLOWING INFECTION WITH BACTERIA OR VIRUS

ORGANISM	<u>D. PNEUMONIAE</u>	<u>P. TULARENSIS</u>	SANDFLY FEVER VIRUS
	Gram + extra-cellular	Gram - intra-cellular	
HOST	Mouse, Rat	Human	Human
CHANGE TIME POSTINFECTION	12-16 hr	3-5 days	3-5 days
α_1	decrease	increase	increase
α_2	increase	increase	<u>a/</u>
β	increase	decrease	--
γ	--	decrease	decrease

a. No change.

mice or rats a significant decrease in α_1 -glycoglobulin occurred which was accompanied by elevations in the α_2 - and β -fractions and no change in the γ -fraction. In tularemia in humans, on the other hand, an increase in α_1 - and α_2 -glycoglobulins and a decrease in the β and γ fractions were observed 3-5 days following infection. These findings may reflect host differences, that is mouse vs. human; pathogen differences, that is the pneumococcus, (a gram (+) extracellular parasite) vs. P. tularensis (a gram (-) intracellular parasite); or differences in experimental design; the studies of pneumococcal infection in mice were specifically designed to study the first 24 hr of infection, whereas most human studies, including ours, rarely include samples obtained more frequently than every 24 hr. That species differences may play a role is suggested by the fact that the serum glycoprotein pattern in normal mice and rats is characterized by a prominent α_1 -glycoglobulin fraction which is not prominent in normal humans but increases after exposure to infection.

Our future plans include studies of more prolonged infection in mice and more careful investigation of the first 24 hr of infection in humans to determine if the changes described are different because of host-parasite differences or are all part of an evolving and dynamic infection-induced disturbance in glycoprotein metabolism. If decreases in α_1 -globulins do occur during the first 24 hr of infection in humans, we would certainly need to reevaluate our present thinking.

Whether an extracellular parasite may induce changes different from those induced by an intracellular parasite is a question which remains to be answered. We are planning to do this in both animals and humans. However, from the data which is available when a bacterial and a viral infection in humans are compared, certain distinguishing characteristics are obvious. Both *P. tularensis* and the virus of sandfly fever are intracellular parasites, yet the bacterial infection is characterized by an increase in both the α_1 - and α_2 -glycoglobulins and a decrease in the β - and γ -fractions whereas the virus infection is accompanied by significant changes in neither the α_2 - or β -glycoprotein fractions. Inasmuch as glycoprotein changes in both the viral and bacterial infection occur at about the same time during infection, that is, accompanying the onset of fever, we may have here the foundation of a simple means of differentiating between bacterial and viral disease. Since our present method requires < 0.1 ml of serum and takes < 3 hr, it is certainly practical. The specificity, however, remains to be substantiated. Lack of change in routine α -globulins and only slight elevation of β -globulin has been reported in viral hepatitis as compared to other acute and chronic infections and inflammatory diseases, but little information is available concerning glycoprotein changes in prospective, carefully controlled viral infections. In addition, glycoprotein changes have been induced by sterile inflammation and have been described in a number of inflammatory diseases such as rheumatoid arthritis and systemic lupus. Future studies will have to document first the specificity of the described changes for infection and then the ability to differentiate between bacterial and viral illness. I would postulate that should glycoprotein changes be specific, such specificity would be limited to early acute infection and would be lost as infection blended into nonspecific host inflammatory responses. That glycoprotein studies may ultimately be used as a means of differentiating viral from bacterial disease is not an unreasonable hypothesis when one considers, for example, the possible role of glycoproteins as receptors on the red blood cell surface for viruses causing hemagglutination such as influenza, mumps, and Newcastle disease viruses as well as their role as hemagglutination inhibitors.

Although the mechanisms for changes in serum glycoproteins remain unexplained, a number of hypotheses have been proposed to explain the elevated levels observed:

1. Glycoproteins are released into the bloodstream from injured, inflamed, or otherwise altered tissue.

2. Glycoproteins arise as a response of the organ to tissue injury or proliferation.

3. Glycoproteins become elevated because certain tissues utilize proteins low in bound carbohydrate, e.g., utilization of albumin may leave the carbohydrate rich fraction.

There have been reports in the literature that certain tumors utilize α_2 -glycoprotein preferentially; this has not yet been demonstrated in infection. Such utilization of a specific glycoprotein fraction might explain the decrease in α_1 -gycglobulin described previously in pneumococcal infection in mice.

Since the liver is the site of almost all glycoprotein synthesis with the exception, perhaps, of γ -gycglobulin, serum glycoprotein changes may reflect infection-induced increases or decreases in hepatic glycoprotein synthesis.

Further insight into mechanisms of glycoprotein changes may have been gained by analysis of the data of Shambaugh and Beisel³⁷ who studied insulin response during tularemia in the same group of patients in which we studied serum glycoproteins. They demonstrated that within 24 hr of the onset of clinical illness, the rate of glucose disappearance from the blood diminished significantly. At the same time, a brisk rise in insulin levels occurred. This suggested a peripheral inhibition of insulin action. The biosynthesis of at least the glucosamine component of the glycoproteins is not affected by insulin deficiency. It is conceivable, therefore, especially since glycoprotein and insulin changes occurred at the same time during infection, that there is a shunting of glucose from its insulin-dependent pathways to the biosynthesis of glycoproteins. This seems reasonable in view of the fact that glycoprotein levels in general are somewhat higher in diabetics. We are in the process of evaluating this hypothesis by studying infection-induced serum glycoprotein changes in alloxan-diabetic mice.

SUMMARY

A reevaluation of infection induced serum glycoprotein changes seems warranted. Early prospective studies suggest that these changes may be dynamic and that determinations of these components early in infection may be sufficiently specific to differentiate between viral and bacterial infection. When considered with the intermediary metabolism of both carbohydrates and proteins, further insight into the pathogenesis of infection may evolve. In addition, since there is some evidence that excess glycoproteins may be deposited in any number of body tissues, e.g., in the blood vessels of diabetics, this may be part of the link between infection and chronic degenerative disease.

LITERATURE CITED

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DISCUSSION

DR. CLUFF: Thank you very much Captain Klainer. Interest in glycoproteins has waxed and waned over the years in a whole host of diseases. I can remember not too many years ago when there was a report not too dissimilar from your own, that the changes in glycoproteins with various diseases might prove to be the most excellent laboratory diagnostic tool in clinical medicine. Further evaluation proved this to be fallacious. You've mentioned the nonspecificity of the reaction, at least in the sense that these changes may be similar to those that have been observed in other diseases. Rather than the diagnostic implication, I'd be more interested in the functions of the glycoproteins relative to the changes that occur in the different components that you have mentioned. What is the difference between α_1 , β , and γ glycoproteins?

DR. KLAINER: Serum glycoproteins have been studied in approximately 68 diseases. The time at which they have been studied is variable or indefinite. As an example, the literature really doesn't tell us whether a rheumatoid arthritis patient in whom serum glycoproteins have been studied, for instance, has been in the hospital a week, 10 days, or 2 months. This has been one of the problems. Reevaluation may be interesting because we have had the opportunity in the Medical Unit to study this prospectively. We know exactly when infection has occurred.

In answer to your second question, there are two ways to study glycoproteins: by the fraction of serum proteins with which they migrate or by their carbohydrate moiety. Many compounds in the body are glycoproteins. These have been characterized more by their carbohydrate moiety than by the fraction of serum proteins with which they migrate. A review article by Spiro, in the New England Journal of Medicine several years ago, subdivided these compounds by their various carbohydrate fractions. Less is known about their migration with protein fractions.

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INTERACTION OF NUTRITION AND INFECTION IN DOGS

Paul M. Newberne, Ph.D.*

During the course of studies designed to investigate the interrelationships of nutrition and infection, it was observed that overfed, obese dogs possessed considerably less resistance to the virus of canine distemper than did either their normally fed or slightly underfed counterparts. We have confirmed and extended these observations through studies designed to elucidate some of the biochemical mechanism associated with the clinical response.

Litter mate beagle dogs susceptible to distemper infection were obtained from a commercial supplier at 5-7 months of age. They were given a highly palatable balanced diet with a meat base, chosen because of its excellent digestibility and because the dogs would consume it in large quantities. An approximate analysis of the diet is given in Table I; on a dry weight basis it supplied 488 kcal/100 gm. When the dogs were permitted ad libitum access to the diet their weights increased by $\geq 40\%$ in a period of 6 weeks. During this conditioning period, baselines were established for the parameters to be measured after exposure to the virus of canine distemper.

TABLE I. APPROXIMATE ANALYSIS OF THE DIET (DRY WEIGHT)^{a/}

INGREDIENTS	%
Crude protein	27.42
Crude fat	16.13
Nitrogen free extract	44.84
Calcium	0.97
Phosphorus	0.97
Ash (total)	8.40
Crude fiber	3.21

a. The diet contained 69% moisture when fed.

A 10% brain suspension containing the Snyder-Hill strain of distemper virus (supplied by Dr. James L. Bittle, Pitman-Moore Virus Research Laboratories, Indianapolis, Indiana) was used for inoculation. Each animal received 0.02 ml/kg of body weight of the suspension intracerebrally (IC); control animals were given an equivalent volume of physiological saline.

* Massachusetts Institute of Technology, Cambridge, Massachusetts, Contract DA-49-193-MD-2560.

Using the technique of Farrell and Ott^{1/}, the inoculum was introduced under anesthesia into the cerebral hemisphere through a sterile opening in the cranium. The virus suspension was delivered from a 1.0-ml glass syringe through a 20-gauge, 1.5-inch needle; distribution of the virus throughout the thickness of the cortex was achieved by slowly withdrawing the needle as the inoculum was discharged. In order to diminish the chance of infection of the control groups, one person tended these animals; no one working with infected dogs entered the room housing the control animals. Confirmation of inoculation and infection was made by histologic examination of tissues.

All dogs were examined twice daily and temperatures recorded; body weight was recorded weekly. Blood samples were obtained by jugular puncture at intervals during the trial and at time of sacrifice. Biochemical determinations were made on either serum or plasma. Urine was measured and recorded as the total daily output; an aliquot was frozen under toluene for N determination.

For fecal collections a food-coloring marker mixed in 1:10 dilution with methyl cellulose was administered orally every 3 days. Three-day fecal pools were homogenized with water in a Waring blender and an aliquot was preserved by freezing. Records were kept on appetite and food consumption, on the general behavior of the animals, and on any symptoms that developed following inoculation. Animals in the first few trials were sacrificed when symptoms of paralytic encephalitis, characteristic of the terminal stages of the infection, were observed.

The caloric content of the food was determined by bomb calorimetry. Total N was estimated in the diet, urine, and feces by the Kjeldahl method. Urinary creatinine was measured by the Folin method, using a Technicon Autoanalyzer (Technicon Co., Inc., Chauncey, N. Y.). Urea and ammonia N were determined by the Conway microdiffusion method. Serum and liver amino acids were determined on the autoanalyzer.

Total serum protein was measured with a refractometer (The American Optical Co., Buffalo, N. Y.) and selected samples were checked by micro-Kjeldahl analysis. Electrophoretic separation of plasma proteins was made on cellulose-acetate strips in Coleman chambers (Gelman Instrument Co., Ann Arbor, Mich.). A barbitone buffer with calcium lactate was used at a concentration of 8.8 gm/L and a current of 1.5 ma was applied for 1.5 hr. Following staining with Ponceau S the strips were scanned in a Beckman analytrol (Beckman Instruments, Palo Alto, Calif.).

Protein-bound iodine (PBI) values were determined by a reliable commercial laboratory (Boston Medical Laboratory, Boston, Mass.). Glucose and cholesterol in serum were determined according to standard techniques.
2,3/ Adrenal corticoid levels in the serum were analyzed by a method which was a slight modification of that used by Daly and Spencer-Peet.^{4/}

The dogs were sacrificed at varying intervals postinoculation. They were placed under deep anesthesia and the cranial cap removed. The cord was then severed at the level of the pons; samples of 3 levels of brain and of the liver were quickly removed, quenched in liquid propane at -175°C, and processed for analysis, or stored for later use. The remainder of the brain and other organs was preserved in neutral formalin or stored at -70°C. Tissues for morphologic study were freeze-substituted; those for histoenzymologic study were cut on the cryostat and treated according to standard histochimical techniques.

Adenosinetriphosphate (ATP) levels were determined by the method of Tal et al.⁵ Total protein, RNA, DNA, and lipids were measured by standard techniques.⁶

The amount of food consumed by the ad libitum (high level) groups averaged 90-100 kcal/kg of body weight per day. Control groups were fed 70-75 kcal/kg of body weight per day; moderate and low level groups were fed 70-75 and 40-50 kcal/kg, respectively. In the later trials the moderate intake group was not used. A separation in body weights of the groups was achieved; the characteristic pattern is shown in Figure 1. Typical body temperature curves are shown in Figure 2. An initial rise in temperature occurred 3-5 days postinoculation; followed by a decrease; the temperature then rose on days 9 and 10 to constitute the characteristic diphasic curve associated with clinical distemper.

Characteristic mortality and average survival times for all trials are shown in Table II. The data clearly indicate the greater susceptibility and shorter survival time of dogs fed the high level of caloric intake and inoculated with distemper virus (86 vs. 26% mortality for dogs in the low fed groups). Animals fed the moderate level of calories and exposed to distemper virus responded in a manner somewhat similar to that observed in dogs fed the high level of calories.

TABLE II. MORTALITY AND SURVIVAL TIMES OF CONTROL AND DISTEMPER-INFECTED DOGS

DIETARY TREATMENT	AVERAGE SURVIVAL TIME (days)	MORTALITY	
		No.	%
Control ^{a/}	--	0/14	--
Low ^{b/}	16	6/23	26
Medium ^{b/}	11	12/18	66
High ^{b/}	7	30/35	86

a. Noninoculated.

b. Inoculated.

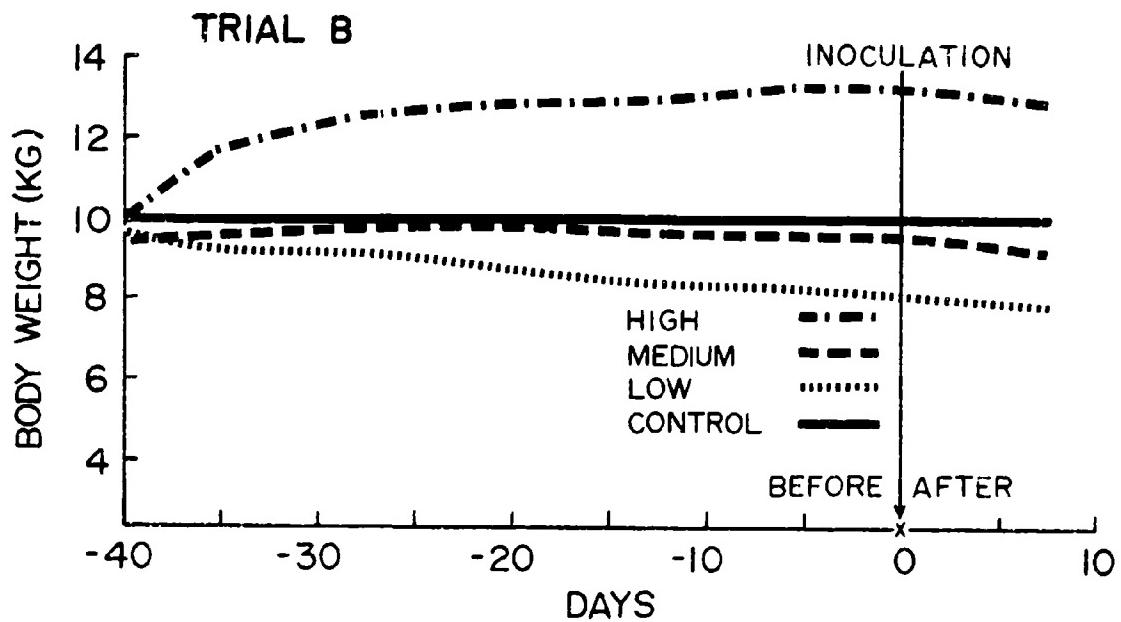


FIGURE I. BODY WEIGHTS OF DOGS FED VARIOUS CALORIE-CONTAINING DIETS.

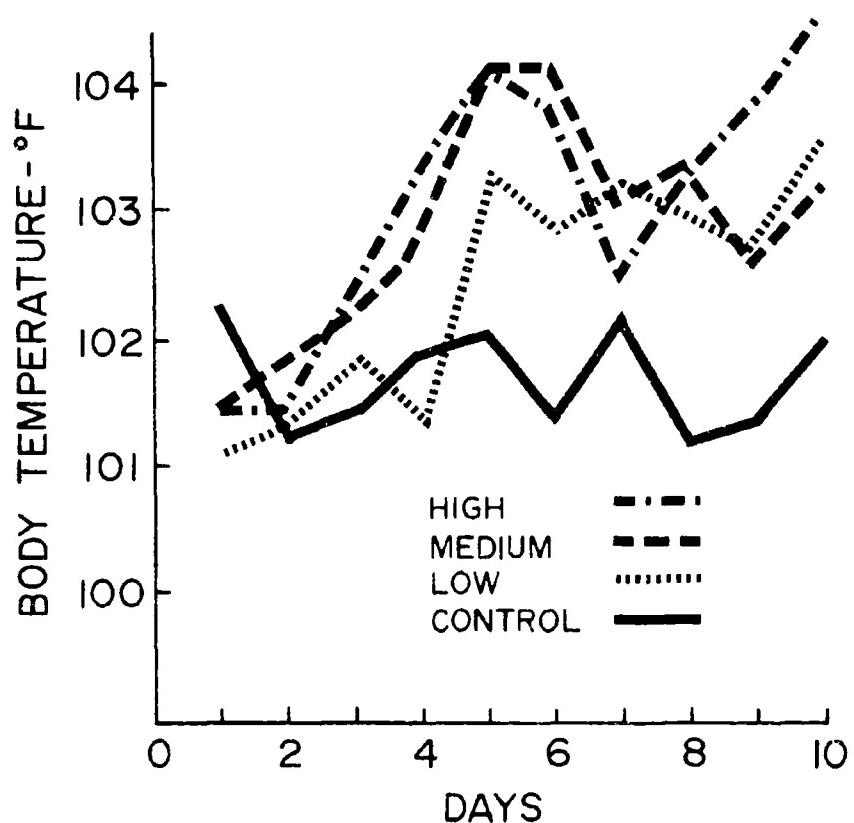


FIGURE 2. EFFECT OF CANINE DISTEMPER INFECTION ON BODY TEMPERATURES OF DOGS FED VARIOUS CALORIE-CONTAINING DIETS.

Total plasma protein was determined prior to and at 2-day intervals after inoculation; electrophoretic separation of plasma protein fractions was done at 10, 14, 22 and 29 days. The important changes had occurred by day 10 and consisted of an increase in total plasma protein and a decrease in the albumin and γ -globulin fractions of infected animals. Table III lists typical results observed in animals from each dietary regimen. In most cases there was an increase in the α_2 globulin fraction; this was a nonspecific reaction since an identical change was observed in 2 noninoculated dogs with transient bacterial infections. The changes in total protein, albumin, and γ globulin were most pronounced in those animals sacrificed because of paralytic encephalitis. Typical electrophoretic profiles (Figure 3) illustrate the peak in the α_2 globulin fraction of infected animals. These observations may be explained in part by the well known depression of the lymphatic system associated with distemper infection. Figure 4 illustrates the depression of follicles and necrosis of lymphatic cells in the spleen of a high fed, infected dog. Glucose 6-phosphatase of the liver and kidney was markedly increased by infection while liver choline esterase was decreased. While these changes were detected in all infected dogs, they were most pronounced in high-fed animals. There was little change in glucose 6-phosphatase in the cerebrum as a result of infection, despite the fact that this was the site of virus inoculation.

Serum neutralizing antibody titers were extremely variable and did not correlate with the clinical course of the disease. Characteristic results are shown in Table IV. Detectable antibody titer and resistance to distemper did not correlate in the dogs in this investigation.

Determinations of PBI yielded interesting and important results. Table V lists results showing that, where sacrifice was necessary because of paralytic encephalitis, there was a sharp drop in PBI values. In most of these cases the drop preceded paralysis by 24-48 hr. An analysis of selected samples of serum showed that the decrease in PBI values was accompanied by a decrease in plasma glucose and, in some cases, by an increase in plasma cholesterol. Histochemical stains revealed a decrease in choline esterase and ATPase and an increase in glucose 6-phosphatase in the fat content of the thyroid of high-fed, infected dogs.

Nitrogen balance studies, in which total N gained or lost after inoculation was measured, revealed wide variations between inter- and intra-groups. Figure 5 compares an overfed animal with a total N loss of about 32 gm at time of sacrifice on day 8 with a low-fed dog. The latter survived despite large losses while the high-fed animal was sacrificed because of paralysis. In general, however, it appeared that most overfed dogs lost a large amount of N in a short period of time and usually succumbed to the infection; low-fed animals often lost as much but did so over a longer period of time and usually survived the infection. Determinations of urinary urea, ammonia, and creatinine did not reveal any consistent alteration in these fractions among the various groups, although there were indications that infection decreased the amount of N excreted as urea in obese animals.

TABLE III. ELECTROPHORETIC PATTERNS OF PLASMA PROTEINS

DOG NO. BY DIET	DAY	PLASMA PROTEINS (gm/100 ml)	Albumin	% Globulin			
				α_1^-	α_2^-	$\beta^- + \phi^-$	γ^-
<u>Control</u>							
5	0	6.0	49.5	9.4	12.4	21.7	7.0
	+10	6.0	50.6	7.4	9.9	24.7	7.4
12	0	5.7	49.6	11.7	9.0	19.9	9.8
	+10	6.1	40.9	7.3	13.8	26.9	11.1
13	0	5.9	48.7	12.2	8.7	21.8	8.6
	+10	6.0	47.0	9.2	9.2	26.2	8.4
<u>Low</u>							
1	0	5.4	45.7	6.8	12.6	26.2	8.7
	+10	5.1	45.4	11.3	10.3	22.7	10.3
6 ^{a/}	0	5.7	54.8	11.1	9.4	16.1	8.6
	+10	7.8	37.5	6.7	18.4	31.4	6.0
<u>Medium</u>							
8	0	5.8	48.3	9.8	10.7	22.3	9.0
	+10	7.3	38.3	8.0	14.5	31.9	7.3
11 ^{a/}	0	5.7	51.1	14.0	9.3	16.3	9.3
	+10	7.6	37.0	6.9	15.7	32.5	7.9
<u>High</u>							
2	0	6.1	54.3	7.0	8.5	22.4	7.8
	+10	6.4	39.8	7.4	14.3	31.1	7.4
9 ^{a/}	0	6.0	51.3	11.5	11.5	18.3	7.4
	+10	8.4	40.7	6.2	19.7	27.0	5.4
14 ^{a/}	0	5.8	52.6	11.9	8.2	21.8	5.5
	+10	7.4	40.5	7.3	17.9	28.5	5.8

a. Sacrificed.

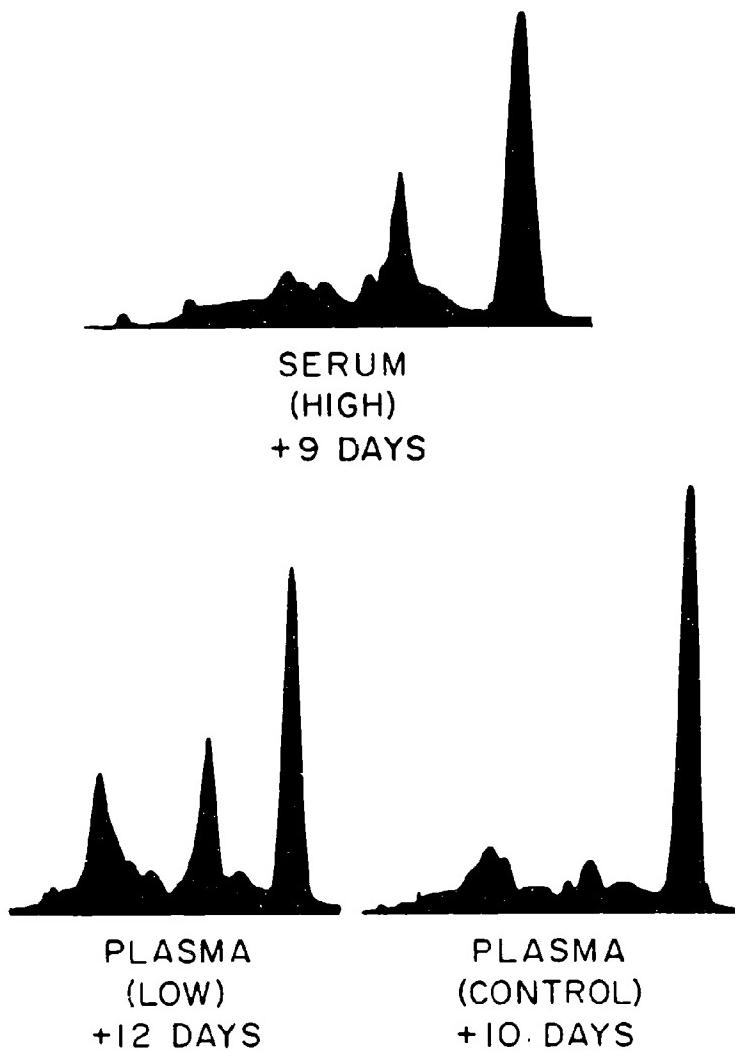


FIGURE 3. ELECTROPHORETIC PATTERNS OF SERUM PROTEINS.

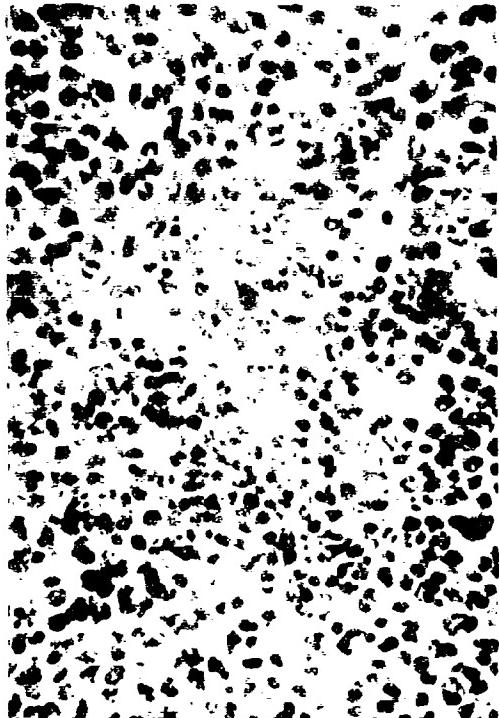


FIGURE 4. SPLEEN FROM DISTEMPER-INFECTED DOG FED A HIGH CALORIE DIET. FOLLICLE SIZE AND CELLULAR DENSITY MARKEDLY INCREASED (LOW POWER) AND NECROSIS AND FRAGMENTATION OF LYMPHOID CELLS (HIGH POWER).

TABLE IV. SERUM ANTIBODY TITERS AND MORTALITY

DIETARY TREATMENT	GROUP	MAXIMUM ANTIBODY TITER	RESPONSE
Low	1	1:10	Survived
	2	1:460	Survived
	3	1:300	Sacrificed
	4	1:30	Survived
Medium	1	1:300	Sacrificed
	2	1:30	Sacrificed
	3	1:300	Survived
High	1	1:30	Sacrificed
	2	1:3,000	Sacrificed
	3	1:3,000	Survived
	4	1:300	Sacrificed

ATP levels were measured in the cerebrum, cerebellum, liver and thyroid of the dogs on day 5 postinoculation (Table VI). Since it would seem that the virus requires a source of energy in order to replicate, it appeared worthwhile to determine whether or not tissues of overfed animals contained quantities of ATP different from those of control or underfed dogs. The data indicate that the group fed the high caloric level had the highest level of ATP in the brain and the lowest level in the thyroid. Liver levels were not markedly different among the various groups. It appeared that preinoculation conditioning had an effect on the response; the 6-week period of conditioning resulted in levels of ATP higher than those in the dogs conditioned only 3 weeks. This occurred despite the fact that the low-fed animals in both conditioned groups weighed about the same at the end of the experiment. It seemed significant that infected animals generally had higher concentrations of ATP than did controls.

Tables VII and VIII and Figure 6 illustrate the influence of infection on liver total protein and nucleic acids at days 3, 5 and 7 postinfection in obese and low-fed dogs. Significant observations include a decrease in liver protein at 3 and 5 days in the obese, infected animals; after 7 days this parameter appeared to stabilize. A significant portion of the liver protein lost consisted of essential amino acids. In contrast to the response of obese dogs, liver total protein in low-fed animals increased at all periods measured. A part of this increase consisted of essential amino acids.

TABLE V. SELECTED DETERMINATIONS FOR PLASMA PROTEIN-BOUND IODINE, GLUCOSE, AND CHOLESTEROL IN CONTROL AND DISTEMPER-INFECTED DOGS

DIETARY TREATMENT	DOG NO.	PBI (μ g/100 ml plasma)	GLUCOSE (mg/100 ml plasma)			CHOLESTEROL (mg/100 ml plasma)			SURVIVAL TIME	
			Pre- inocu- lation	Terminal inocu- lation	% Change	Pre- inocu- lation	Post- inocu- lation	% Change		
Control	2	3.8	3.8	--	57.4	91.4	+59.2	300	283	371 Survived
High	7	3.6	1.8	-50.0	104.1	74.4	-28.5	325	395	510 Sacrificed 10 days
High	8	4.2	2.2	-47.6	80.3	57.4	-28.5	290	477	430 Sacrificed 11 days
Low	16	2.4	1.8	-25.0	87.1	53.1	-39.0	413	402	416 Sacrificed 10 days
Low	13	2.6	1.8	-30.8	75.4	55.2	-26.8	310	370	257 Sacrificed 12 days

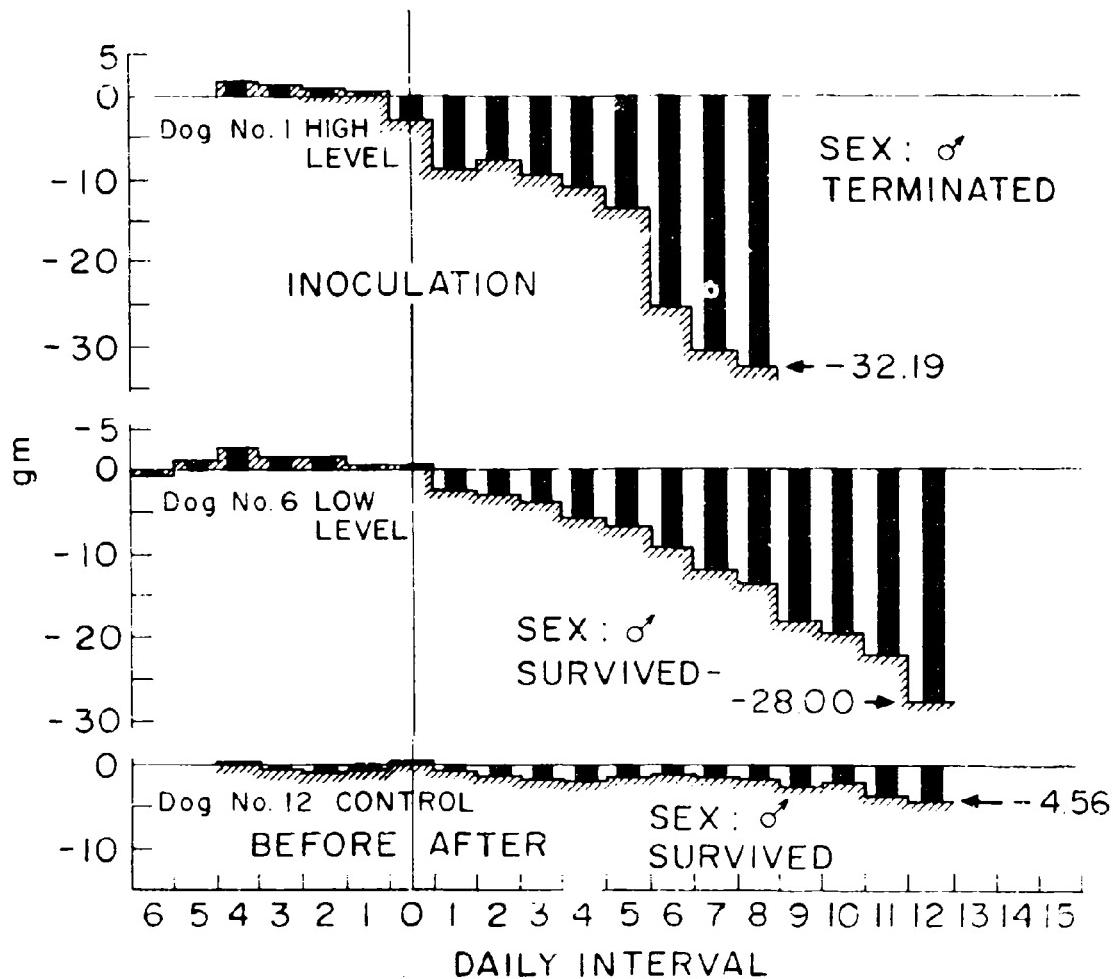


FIGURE 5. EFFECT OF DISTEMPER INFECTION ON CUMULATIVE NITROGEN BALANCE OF DOGS FED LOW AND HIGH CALORIE DIETS.

TABLE VI. ADENOSINETRIPHOSPHATE (ATP) LEVELS OF SELECTED TISSUES FROM DISTEMPER-INFECTED AND CONTROL DOGS^{a/}

TISSUE	ATP μ MOLES/GM					
	Distemper-Infected				Control	
	Conditioned 6 weeks		Conditioned 3 weeks		Conditioned 6 weeks	
	High ^{b/}	Low	High	Low	High	Low
Cerebrum	47.0	26.0	17.4	9.8	37.7	23.9
Cerebellum	47.4	26.0	28.9	18.9	43.2	41.4
Liver	5.5	6.3	2.4	1.8	11.0	6.3
Thyroid	1.3	5.9	1.8	3.4	7.4	12.8

a. Mean of 3/group.

b. High or low calorie diets.

TABLE VII. LIVER TOTAL PROTEIN LEVELS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	TOTAL PROTEIN gm% BY DAY POSTINOCULATION			
	Preinoculation	3	5	7
High-fed				
Control	14.7	14.5	13.8	15.8
Infected	--	11.4	11.5	15.3
Low-fed				
Control	13.2	12.7	12.4	14.6
Infected	--	13.6	14.2	15.9

TABLE VIII. LIVER NUCLEIC ACID LEVELS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	NUCLEIC ACIDS mg/100 gm BY DAY POSTINOCULATION				
	Preinoculation	3	5	7	
High-fed		RNA-P			
Control	80.4	66.6	76.2	98.5	
Infected	--	72.3	36.9	67.8	
Low-fed		DNA-P			
Control	72.3	63.4	101.9	58.9	
Infected	--	58.9	55.7	68.1	
High-fed		RNA/DNA Ratio			
Control	29.0	33.8	24.4	28.7	
Infected	--	34.5	20.5	23.0	
Low-fed		RNA/DNA Ratio			
Control	30.2	35.9	28.7	26.0	
Infected	--	36.4	28.0	28.2	

TABLE IX. LIVER LIPID LEVELS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	LIPIDS gm% BY DAY POSTINOCULATION			
	Preinoculation	3	5	7
Total lipids				
High-fed				
Control	18.3	16.5	13.9	24.4
Infected	--	14.1	16.8	28.1
Low-fed				
Control	18.2	17.2	16.8	20.6
Infected	--	19.5	19.9	20.4
Phospholipids				
High-fed				
Control	8.64	8.02	7.46	10.44
Infected	--	8.00	8.92	11.08
Low-fed				
Control	9.11	9.79	7.97	9.58
Infected	--	9.07	9.70	10.64
Cholesterol				
High-fed				
Control	1.18	1.06	1.17	1.31
Infected	--	1.01	1.27	1.34
Low-fed				
Control	1.17	1.10	1.10	1.32
Infected	--	1.05	1.40	1.17

Liver RNA decreased in both obese and low-fed dogs as a result of infection (Table VIII). Seven days postinfection the low-fed animals had a slight increase in liver RNA. There were no significant alterations in liver DNA in either of the groups, a factor that accounted for the general but slight decrease in the RNA/DNA ratio. A better illustration of the effects of infection on these parameters is shown in Figure 6. The graphs show the deviation of values as percentage change in infected animals compared to their noninfected controls.

Table IX and Figure 7 show that, except for an initial drop in the obese dogs on day 3, infection resulted in an increase in liver total lipids in both groups of animals. Although a good portion of the increase in lipids was in the form of triglyceride, a significant amount of it in both groups was in the form of phospholipids, with a lesser fraction as cholesterol in the obese animals.

Serum proteins have been referred to previously (Table III). Table X indicates that the significant change was an increase in serum total protein in both groups at all periods except on day 7, at which time the values for the low-fed dogs had returned to control levels.

TABLE X. SERUM TOTAL PROTEIN LEVELS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	TOTAL PROTEIN gm% BY DAY POSTINOCULATION			
	Preinoculation	3	5	7
High-fed				
Control	6.56	6.26	6.40	6.01
Infected	6.76	6.71	6.91	7.40
Low-fed				
Control	5.85	5.57	5.67	6.27
Infected	6.16	6.21	6.39	6.23

Serum total lipids fluctuated in both groups, with obese, infected animal maintaining concentrations near control levels (Table XI and Figure 8). The low-fed, infected animals registered a sharp increase in total lipids on day 7. A pattern similar to that observed in the liver was seen in serum phospholipids, with the increase in the low-fed animals occurring earlier than in obese animals. Serum cholesterol in the low-fed, infected animals showed an initial increase but returned to control levels by day 7. The obese animals tended to have less serum cholesterol at all periods measured.

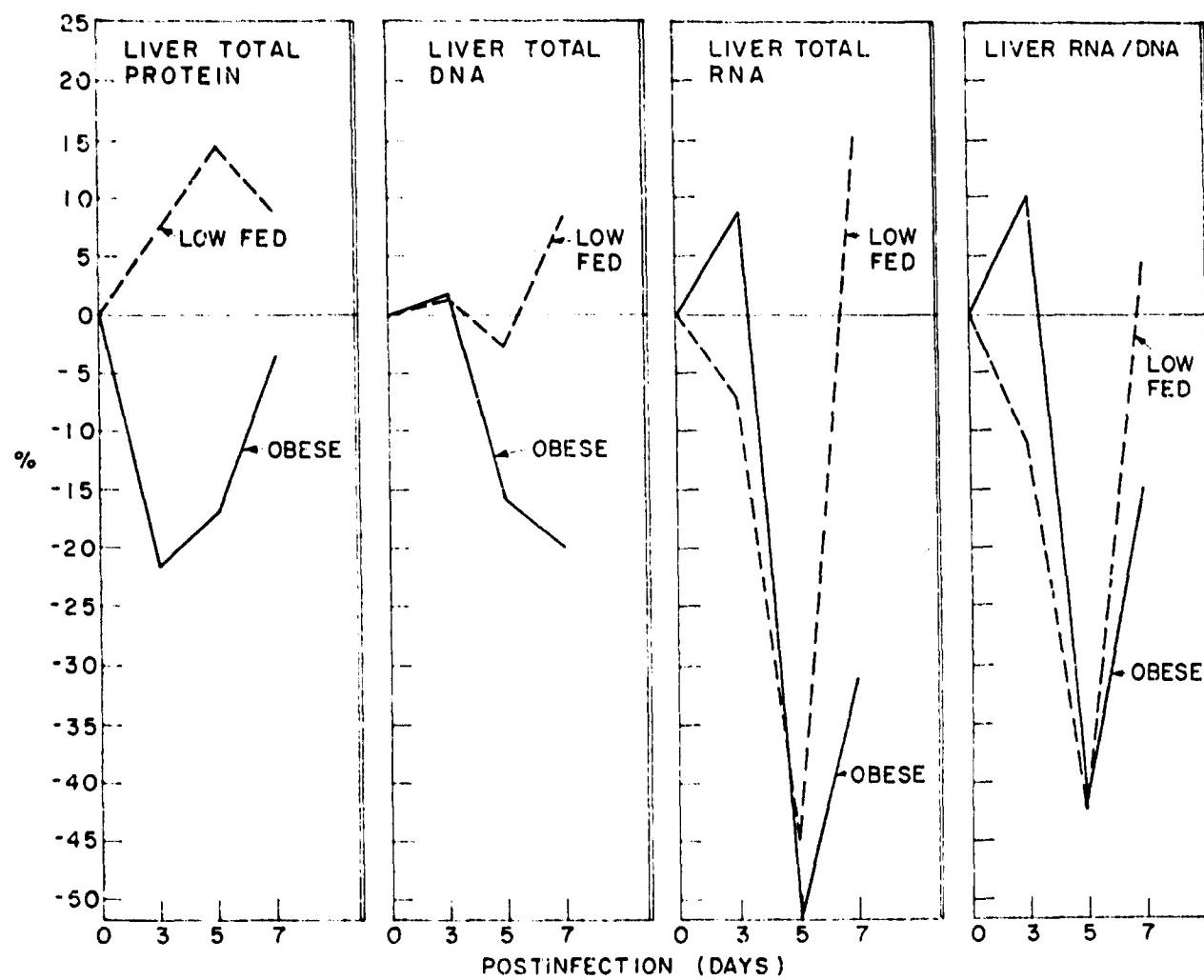


FIGURE 6. LIVER TOTAL PROTEIN AND NUCLEIC ACIDS IN DOGS FOLLOWING INFECTION WITH DISTEMPER VIRUS.

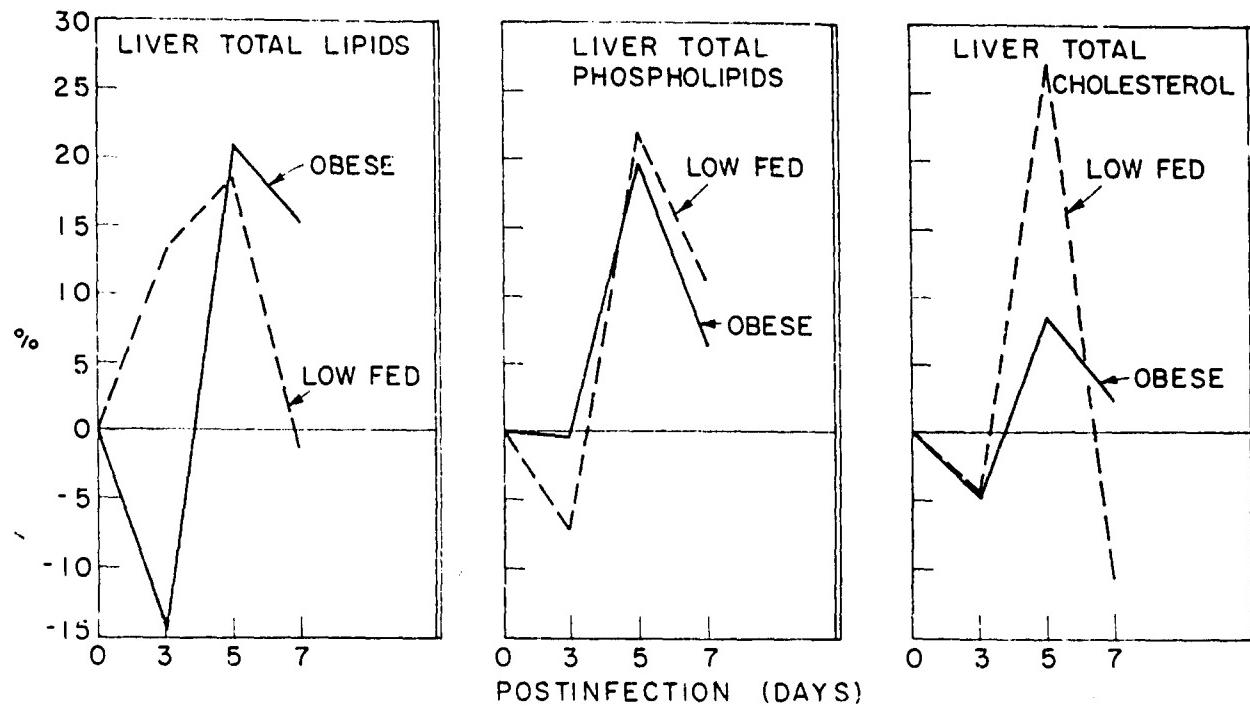


FIGURE 7. LIVER LIPIDS IN DOGS FOLLOWING INFECTION WITH DISTEMPER VIRUS.

TABLE XI. SERUM LIPID LEVELS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	LIPIDS mg% BY DAY POSTINOCULATION			
	Preinoculation	3	5	7
<u>Total lipids</u>				
High-fed				
Control	1525	1420	1423	1328
Infected	1381	1422	1452	1311
Low-fed				
Control	1146	1403	1301	1299
Infected	1342	1266	1328	1420
<u>Phospholipids</u>				
High-fed				
Control	464.3	357.5	340.0	357.5
Infected	439.3	332.5	462.8	437.5
Low-fed				
Control	275.3	298.8	381.5	365.8
Infected	366.0	442.3	408.0	412.8
<u>Cholesterol</u>				
High-fed				
Control	284.0	305.0	297.0	327.0
Infected	246.0	270.0	274.5	280.3
Low-fed				
Control	232.8	207.2	249.3	242.3
Infected	218.7	259.0	266.0	242.3

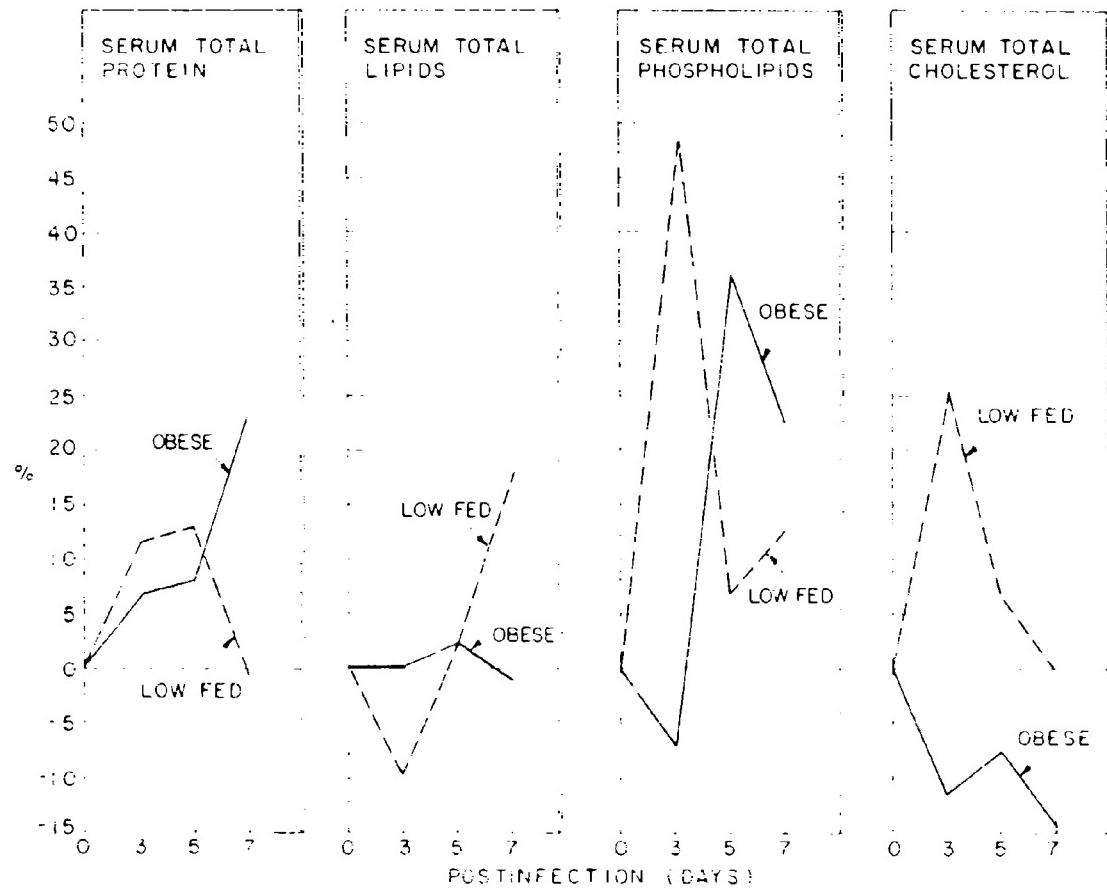


FIGURE 8. SERUM PROTEIN AND LIPIDS AFTER INFECTION WITH DISTEMPER VIRUS.

Results of free amino acid analyses in serum and liver are shown in Tables XII-XIV. Changes, often very great, in the concentration of individual free amino acids in the liver were not always reflected in serum concentrations. In obese, infected animals sharp decreases in the liver concentrations of threonine, valine, methionine, serine, and α -amino-butyric acid, as a result of the infection, were not accompanied by remarkable alterations in serum concentration of these amino acids. Lysine was increased in both the liver and serum at 3 days but tended to stabilize on day 5. In general, it appeared that the obese, infected animal was unable to retain liver protein (Table VII) and that an important fraction of this protein loss consisted of the critical essential amino acids. The reverse was true in the low-fed, infected dogs; they not only retained liver protein but were able to increase the concentration of a number of the essential amino acids. Tables VII and XII-XIV show that the obese, infected dog maintained serum levels of amino acids at the expense of the liver, particularly during the early incubation period of infection, while the low-fed animals conserved liver concentrations by reducing serum levels of some individual amino acids. The essential/nonessential (E/N) ratios (Table XIV) further illustrate this point.

An additional interesting observation was the tendency for the phenylalanine/tyrosine ratio to increase following infection in obese dogs (Table XV). Although not experimentally substantiated, this observed alteration may indicate a block in the enzyme phenylalanine hydroxylase with consequent failure to convert phenylalanine to tyrosine.

Results of determinations of protein, nucleic acids, and lipids of the cerebral hemisphere are shown in Tables XVI and XVII and in Figure 9. The increase in cerebral RNA and DNA in obese, infected animals differed slightly from the response in low-fed animals, in which the RNA was decreased. It is difficult to explain the change in RNA but the change in DNA probably resulted from the increase in glial elements concomitant with encephalitis. The most interesting change in cerebral lipids was the increase in phospholipid and cholesterol content in obese animals following infection, a finding paralleling to some degree the change in these parameters in the liver and serum.

Table XVIII illustrates the decrease in serum PBI levels and the increase in circulating serum cortisol in obese, infected animals. Except for the rather large (in comparison to obese, infected animals) percentage decrease in insulin, the same parameters varied only slightly in the low-fed, infected dogs. However, the markedly lower concentration of insulin in low-fed animals must be considered in attempting to evaluate this observation. These findings have been confirmed and partially extended; they indicate clearly that infection of overfed, obese animals results in a more pronounced change in thyroid and adrenal secretions.

TABLE XII. DISTRIBUTION OF SELECTED AMINO ACIDS OF OBESE CONTROL AND DISTEMPER-INFECTED HIGH-FED DOGS

ACID	AMINO ACIDS IN							
	Serum μ moles/100 ml				Liver μ moles/100 gm			
	3 days	5 days	3 days	5 days	Con	Inf	Con	Inf
	Con ^a /	Inf	Con	Inf	Con	Inf	Con	Inf
Threonine	45	41	43	21	93	55	146	133
Valine	16	17	21	20	223	49	177	77
Leucine	10	11	14	14	45	56	124	85
Tyrosine	7	5	8	5	16	24	126	34
Phenylalanine	5	6	8	10	24	32	121	40
Lysine	7	13	20	16	28	61	102	114
Methionine	--	--	--	--	36	17	36	25
Serine	21	21	33	20	246	75	122	104
Glutamic	19	23	27	27	692	753	963	592
Glycine	19	17	23	16	69	213	245	253
Alanine	49	39	52	33	158	247	457	476
α -NH ₂ -butyric	5	3	3	4	312	46	260	82

a. Con = control; Inf = infected.

TABLE XIII. DISTRIBUTION OF SELECTED AMINO ACIDS OF LOW-FED CONTROL AND DISTEMPER-INFECTED LOW-FED DOGS

ACID	AMINO ACIDS IN							
	Serum μ moles/100 ml				Liver μ moles/100 gm			
	3 days		5 days		3 days		5 days	
	Con ^a /	Inf	Con	Inf	Con	Inf	Con	Inf
Threonine	43	26	35	15	65	115	127	86
Valine	13	10	21	16	28	50	80	58
Leucine	9	6	14	12	36	38	100	73
Tyrosine	4	3	6	5	14	20	39	35
Phenylalanine	5	4	6	8	23	29	40	33
Lysine	6	9	22	14	37	52	90	70
Methionine	--	--	--	--	11	10	30	27
Serine	35	11	29	16	162	59	127	72
Glutamic	27	13	27	20	666	506	511	35
Glycine	24	11	23	14	133	117	195	165
Alanine	36	34	61	26	219	240	465	33
α -NH ₂ -butyric	4	2	3	2	24	6	30	15

a. Con = control; Inf = infected.

TABLE XIV. DISTRIBUTION OF ESSENTIAL AND NONESSENTIAL AMINO ACIDS IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	AMINO ACIDS BY DAY			
	Serum μ moles/100 ml		Liver μ moles/100 gm	
	3	5	3	5
Essential (E)				
High-fed				
Control	103.9	134.5	4.77	9.26
Infected	104.4	104.0	3.21	5.70
Low-fed				
Control	90.2	121.5	2.27	5.82
Infected	64.5	84.0	3.27	6.14
NonesSENTIAL (N)				
High-fed				
Control	152.4	214.3	15.3	18.3
Infected	149.4	167.6	14.0	14.4
Low-fed				
Control	163.4	216.6	12.5	13.4
Infected	86.8	133.3	9.8	3.4
E/N Ratio				
High-fed				
Control	0.69	0.63	0.31	0.51
Infected	0.70	0.62	0.23	0.40
Low-fed				
Control	0.55	0.56	0.18	0.44
Infected	0.74	0.63	0.33	1.81

TABLE XV. PHENYLALANINE/TYROSINE RATIO IN CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	RATIO BY DAY			
	Serum		Liver	
	3	5	3	5
High-fed				
Control	0.75	0.93	1.50	0.97
Infected	1.34	1.86	1.34	1.18
Low-fed				
Control	1.33	1.12	1.65	1.02
Infected	1.34	1.68	1.47	0.93

TABLE XVI. DISTRIBUTION OF TOTAL PROTEIN AND NUCLEIC ACIDS IN CEREBRUMS OF CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	TOTAL PROTEIN	RNA-P	DNA-P	RNA/DNA RATIO
	gm/100 ml	mg/100 ml	mg/100 ml	
High-fed				
Control	6.0	8.2	8.3	0.98
Infected	6.5	9.8	9.0	1.10
Low-fed				
Control	6.3	13.8	7.9	1.75
Infected	5.4	10.1	9.3	1.10

TABLE XVII. DISTRIBUTION OF LIPIDS IN CEREBRUMS OF CONTROL AND DISTEMPER-INFECTED DOGS

TREATMENT	LIPIDS gm% BY DAY POSTINOCULATION			
	Preinoculation	3	5	7
<u>Total Lipids</u>				
High-fed				
Control	48.8	48.9	49.4	48.1
Infected	--	51.2	50.8	46.1
Low-fed				
Control	49.1	49.6	47.5	50.1
Infected	--	48.3	47.0	50.0
<u>Phospholipids</u>				
High-fed				
Control	24.10	26.50	23.05	22.74
Infected	--	27.12	22.95	25.62
Low-fed				
Control	24.82	26.02	23.03	25.42
Infected	--	25.79	22.35	25.74
<u>Cholesterol</u>				
High-fed				
Control	9.73	10.03	9.9	9.26
Infected	--	10.42	10.9	9.37
Low-fed				
Control	10.10	10.13	10.0	10.16
Infected	--	9.48	9.6	9.26

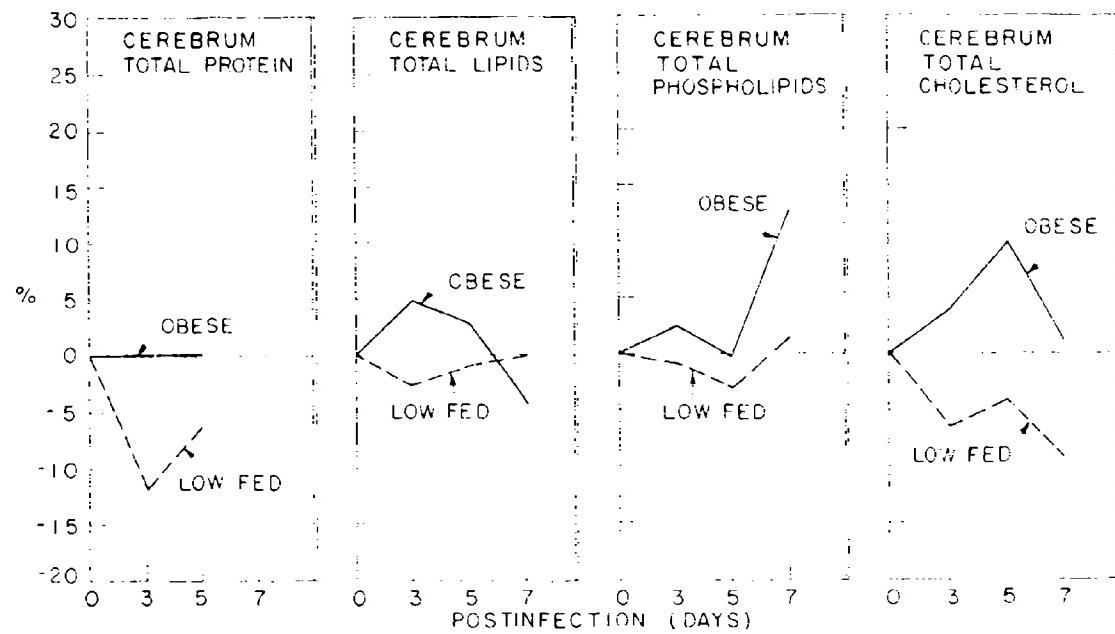


FIGURE 9. CEREBRAL PROTEINS AND LIPIDS AFTER EXPOSURE TO DISTEMPER VIRUS.

TABLE XVIII. SELECTED BLOOD CHEMISTRIES FOR DISTEMPER-INFECTED DOGS^a/

	INSULIN μcount	PBI μg/100 ml	CORTISOL	BLOOD SUGAR mg/100 ml
High-fed				
Control	14.0	3.7	5.5	82
5 days	9.5	2.8	18.5	60
% change				
	-32	-24	+236	-27
Low-fed				
Control	4.0	2.6	9.0	72
5 days	1.0	2.5	12.0	74
% change				
	-75	-4	+33	+3

a. Average 2/value.

Preliminary determinations indicate a difference in the delicate balance of various hormonal secretions of the overfed and underfed distemper-infected animals. There appears to be a higher serum content of insulin in overfed animals; distemper virus infection results in a significant decrease in serum content of insulin whether the animal is overfed or underfed. PBI levels decrease as a result of infection in overfed animals but there is no change in the PBI serum concentration of underfed dogs; the blood sugar follows a similar pattern. The remarkable increase in circulating cortisol levels in the overfed animals is of interest. Underfed dogs appear to develop a tolerance to the specific stress whereas overfed animals do not have this facility and, when exposed to distemper virus, react in a more acute manner. Although these determinations are preliminary and limited in number, they indicate the complex nature of the response of an animal to the stress of an infectious agent and clearly point to a need for investigations into this very important aspect of the relation of nutrition to infection.

SUMMARY

In order to test the influence of caloric intake on resistance to infection purebred beagles were divided into groups and fed a balanced diet supply either 90-100, 70-75, or 40-50 kcal/kg of body weight per day. The moderate level of caloric intake was omitted following the preliminary

experiments. High caloric intake resulted in obesity after 5 or 6 weeks on the diet; the low-intake animals lost about 1 kg during the 6-week period. Basal values were determined for N balance and blood chemistry during equilibration; the dogs were then exposed to distemper virus by IC inoculation. N balance and blood chemistries were made at intervals. Paralytic encephalitis was chosen as the point at which animals were to be sacrificed. High-fed, obese dogs had 86% mortality, normally-fed 66% and low-fed, 26%. Cumulative N loss generally was greater and occurred in a shorter period of time in the high-fed group; however, a comparable loss was observed over a more extended period of time in some low-fed animals which survived the disease. Clinical response was severe in the high-fed group and most of these animals developed paralytic encephalitis in 8-10 days. In general, the reaction of the group on the moderate intake tended to resemble that of the high-level group. Serum antibody titers did not correlate with resistance to the virus or with survival. There was an increase in the α_2 globulin fraction of serum protein (associated with, but not specific for, distemper infection). Serum PBI decreased sharply while serum cortisol increased with infection; this was accompanied by a decrease in plasma glucose.

High-fed dogs lost a significant quantity of protein from the liver, resulting in a pronounced decrease in some of the essential amino acids. In contrast, low-fed dogs retained liver protein following infection and actually increased levels of some of the essential amino acids. In general, the serum concentrations of amino acids did not reflect the liver concentrations.

Although there were changes and fluctuations in the lipid content of the serum, liver, and brain, the salient feature was the increase in phospholipid levels which appeared to be a consequence of infection in all groups of animals.

Obese, infected animals had higher concentrations of ATP in the brain than did underfed counterparts. Preliminary results indicate that obesity has a profound effect on the response of the endocrine system of the dog infected with distemper virus.

It is concluded that obese dogs have less resistance to distemper infection and that this decreased resistance is related in some obscure way to protein metabolism and hormone balance.

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DISCUSSION OF SECTION V

Charles L. Wisseman, Jr., M.D.*

DR. WISSEMAN: It is important, from time to time, to attempt to correlate diverse findings from different types of studies. There is one point that I would like to develop here. I am sure that the massive number of changes in the serum and body responses during infection, which appear totally incomprehensible at present, are important, possibly reflecting important processes in pathogenesis of infection. As I sat here listening to a number of different papers on amino acid levels in the blood after infection, on temperature responses, on protein synthesis, on effects of nutrition on virus infections, and on the response of ribosomes to protein synthetic activity, an idea began to emerge from the pattern formed by this group of studies. The influence of nutrition on viral infection has been observed for a long time. Dr. Newberne's presentation today is consistent with some of the observations that have been made in the past. It has been a little puzzling as to why an undernourished animal might resist a virus infection more readily than either the fully nourished control or one that is somewhat overnourished. It seems unlikely that simple changes in the levels of amino acids and nutrients which are consistent with the survival of these cells involved in an infectious process would alter very markedly the replication of a virus within those same cells. This might not be true of agents like rickettsiae which occur inside of cells but which seem to be much more directly dependent upon the level of the nutrients in the external medium than most of the small viruses are. We do know, however, that there are certain factors that influence the absorption of virus onto those cells - some amino acids, some divalent cations, etc.

Another thing that especially struck me was the remarkable influence of stimuli (temperature, nutrition, etc.) on the status of the intracellular structures, i.e., not only do particles like ribosomes decrease in number, but they change in their capacity to synthesize things. Now if, with viral infections, the mode of replication does depend upon a state of host cell enzyme systems and organelles (e.g., ribosomes in the synthesis of protein antigen), it is entirely conceivable that, with a significant decline in the number of these functional particles or with an alteration in their function (as Dr. Young shows), the rate of virus synthesis might well be remarkably affected if not completely inhibited. Certainly if one alters the rate of virus replication relative to the rates of response of host defense mechanisms, one might alter markedly the outcome of an infectious process. This concept which has come out of the accumulation of different studies presented here today would seem worthy of further investigation.

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DR. CLUFF: I'd like to ask a question which is relevant to what Dr. Weissman was referring to. Obviously these studies are directed toward studying complex biochemical and metabolic changes associated with infectious disease. It is perfectly clear that many of these changes are interrelated. Some effort has been made to demonstrate interrelationships by observations on the endocrine and metabolic events that transpire. I wonder if a common mechanism may be found whereby these various studies can be interrelated? I think this is virtually impossible to accomplish by hand. One can attack an identifiable simple single problem at one time, but it seems to me that there might be some advantage in asking the question as to whether or not some of these studies that are being done individually should somehow or other be programmed for interrelationships. I don't know how anyone can do this except in a computer. Has any thought been given to this? I don't know who can answer this, maybe Dr. Beisel can.

DR. BEISEL: In the Medical Unit we have attempted to correlate as many of these divergent concepts as possible, to keep in communication with the people doing studies of this sort. In some instances, correlative studies have been accomplished in a single experimental animal model. For instance, partially completed at the moment is a study done with Dr. Squibb's Newcastle disease model in chickens, measuring sequential changes in hepatic PP and EP as well as simultaneous studies of the amino acid changes. We have tried to correlate findings in the same animals. Other parallel studies involve a different infectious model or an attempt to see if changes may be due to differences between species.

We have subjected several studies to computer analysis. To subject the entire sum of knowledge about host response into a single computerized system would appear to require an exceedingly massive preconceived prospective study. Each time a new variable was introduced into such a study, one might have to double the number of animals in the study to achieve significance, using an Analysis of Variance approach. This would get to rather monumental proportions just to accomplish the work, and such an approach would perhaps be largely busy work. To me, specific, well designed, key studies seem more useful at the present time to investigate leads that look like they might be of fundamental importance.

DR. T. E. WOODWARD: I think, Dr. Cluff, the purpose of meetings of this type is to discuss the facts, to call the leads, the ideas, and hope that someone has been stimulated intellectually to apply them in a proper way. This is actually what Colonel Beisel implied just a few minutes ago. Rather than go off in ten different directions, take a stand with a suitable host. It is very clear that man seems to be the host that we're interested in. When we can use him and use him safely (and it appears that we are able to use him safely, but should do so only when there is a need to use *Homo sapiens*), then we're going to make some sense with our studies.

DR. CLUFF: My point was not to be critical. My point was to offer a constructive suggestion. We heard presentations dealing with changes in proteins, lipids, hormonal secretion, insulin activity, carbohydrate metabolism, etc. Now these may not all be going on simultaneously in the same group of volunteers. There may be, however, interrelationships which our limited knowledge do not allow us to identify. And my only point is this: it might conceivably be useful, when studies are being done in man and a variety of different types of observations are being made, to computerize results in such a way that interrelationships might be identified which otherwise would be missed.

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SECTION VI

BACTERIAL TOXINS

MODERATOR: Dr. J. Vernon Knight

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INTRODUCTION TO SECTION VI

J. Vernon Knight, M.D.*

The sessions in the past two days have dealt in considerable detail with the response of a host to infection, without much recognition of the fact that an infectious agents produces not a single stress but multiple stresses in varying combinations. Some reference has been made earlier to antitoxin, but this afternoon, the program is entitled "Bacterial Toxins," giving recognition to the fact that there are specific components elaborated by microorganisms in the course of an infectious process which might provide more specific answers. If we take all the variables that were brought out earlier in the meeting and then multiply those by the toxic variables which can be separated from the etiologic agents themselves, we would reach some figure that is reminiscent of Dr. Cluff's remark concerning the need for a computer to help assess the various interrelationships present.

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EFFECT OF SPLENECTOMY ON PYROGENIC TOLERANCE TO BACTERIAL ENDOTOXIN

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The capacity of the reticuloendothelial system (RES) to clear and inactivate circulating gram-negative bacterial endotoxin appears to represent the major defense of the normal mammalian host following its initial intravenous (IV) challenge. The evidence rests primarily upon the marked increase in endotoxin toxicity after RES blockade of the normal animal with colloidal particles,^{1,2/} upon the ability of the normal RES to inactivate endotoxin,^{3,4/} and upon the RES localization of IV injected radioactively labeled endotoxin.^{5,6/} The reticuloendothelium of the spleen appears to contribute minimally to this normal defense; no enhancement of endotoxin-induced mortality or pyrogenicity follows splenectomy,^{7,8/} and less than 3% of the labeled endotoxin is fixed within this organ.^{5,6/} These findings are in keeping with earlier studies on the quantitative distribution of the RES among the various organs. In contrast to its relatively minor reticuloendothelial contribution, the spleen plays a major role in the production of specific circulating antibodies following the IV injection of particulate antigens, including bacterial endotoxins.^{8,10-14/} If the pyrogenic tolerance which develops after repeated injections of bacterial endotoxin were based primarily upon a generalized and nonspecific enhancement of the normal RES phagocytic and detoxifying activities, then the splenectomized host should exhibit minimal impairment in ability to acquire such tolerance to endotoxin entails 2 distinct immunological mechanisms, i.e., an early and transient desensitization at the cellular level and a subsequent elaboration of circulating antibody which reacts with common toxophore groupings responsible for endotoxin pyrogenicity thereby assisting the RES in clearance and destruction of the molecule,^{15,16/} then splenectomy should not impair the early phase of pyrogenic tolerance but should significantly retard the later phase when circulating antibody titers normally increase exponentially. Moreover, this late retarding effect should correlate with impaired production of the humoral factors capable of transferring pyrogenic tolerance. In addition, ablation of other segments of RES so as to evoke deficits in phagocytic activity comparable to that following splenectomy, without the associated impairment of antibody production, should inhibit neither the early nor the late phases of pyrogenic tolerance. The present studies in the rabbit and in man demonstrate that precisely such sequences of events take place.

Effect of Splenectomy on Early and Late Phases of Pyrogenic Tolerance in the Rabbit.--An initial IV dose of Escherichia coli endotoxin which induced fever within the sensitive dose range (0.05 µg/kg) revealed no significant differences in the febrile responsiveness of sham-operated and splenectomized rabbits on day 1 (Figure 1). Upon retesting on day 2

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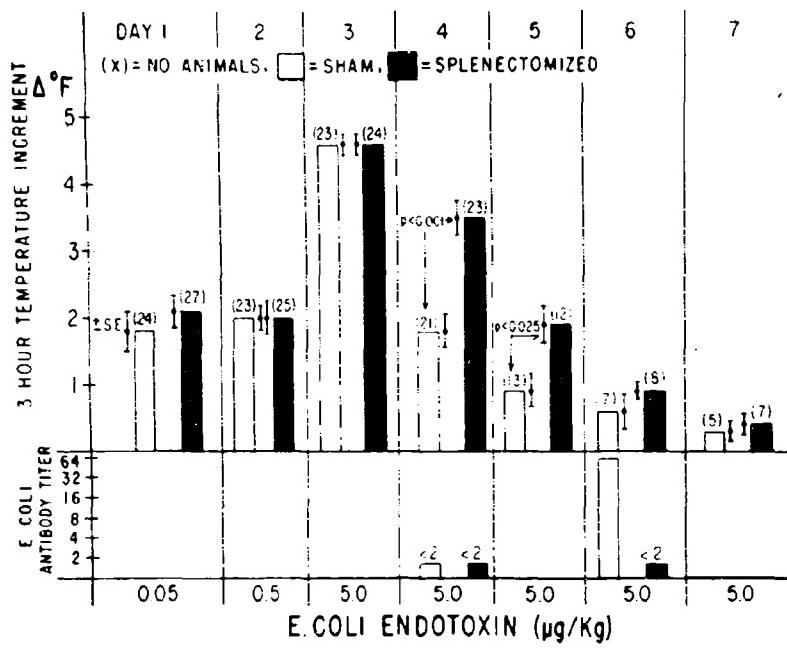


FIGURE I. EFFECT OF SPLENECTOMY ON TOLERANCE AND BENTONITE-FLOCCULATING ANTIBODY AFTER DAILY ENDOTOXIN INJECTIONS.

with a 10-fold larger dose, a significant but equal degree of tolerance was evident in both groups, the mean febrile responses being comparable to those of the day-1 reaction. Retesting with an additional 10-fold increment on day 3 again revealed no differences in pyrogenic reactivity of the splenectomized and sham-operated animals. No effect of splenectomy was therefore evident during the initial 48-hr phase of endotoxin tolerance.

Commencing after 72 hr (onset, day 4), however, a remarkable difference in rate of acquisition of pyrogenic tolerance became discernible, with a highly significant delay in the splenectomized group (Figure 1). In addition, a divergence in ability to elaborate anti-"*O*" antibody became apparent, the splenectomized animals failing to develop detectable antibody titers while the sham-operated animals developed increasing levels.

Transfer of Pyrogenic Tolerance with Plasma from Splenectomized Donors.-- The transfer of pyrogenic tolerance by plasma obtained from splenectomized and sham-operated animals receiving daily IV injections of *E. coli* endotoxin, according to the dose schedule outlined in Figure 1, is depicted in Figure 2; 10 ml/kg of plasma obtained from splenectomized animals on day 4 (24 hr after the 3rd daily endotoxin injection) failed to transfer any detectable protection, whereas similar quantities of plasma from sham-operated controls transferred modest but significant protection ($p < 0.01$). Of special interest, such protection could no longer be transferred when the plasma was obtained on day 4.2, 5 hr after the administration of an additional 5.0 μ g/kg *E. coli* endotoxin (Figure 2). By day 6 (24 hr after the 5th daily injection), 10 ml/kg plasma from both the splenectomized and sham-operated animals transferred excellent pyrogenic tolerance, and although the plasma from splenectomized donors was less efficacious, statistically significant differences were no longer readily obtained. By day 8, no differences were discernible between the high levels of passive protection afforded by 10 ml/kg plasma from splenectomized and sham-operated controls. Anti-"*O*" antibody titers remained undetectable in the serum of the splenectomized animals while progressive increases developed in the sham-operated group beginning at 96 hr and attaining mean levels of 1:246 by day 8.

Effect of Partial Hepatectomy on Pyrogenic Tolerance in the Rabbit.-- The residual impairment of reticuloendothelial phagocytic function 3-5 weeks after removal of the main liver mass of the rabbit, as determined by the uptake of IV injected radioactive colloidal Au¹⁹⁸, is shown in Figure 3. Although approximately 75% of the total liver tissue was initially resected, functional restoration of the RES was so rapid that depletion of reticuloendothelium was evident only by slight retardation in the rate of blood clearance of the colloidal Au¹⁹⁸ and by a 5% increase in the hepatic contribution to the total uptake of injected colloid. This latter difference, however, was significant ($p < 0.02$). Since the splenic contribution to the total uptake of the injected Au¹⁹⁸ amounted to only 1% (Figure 3) it is apparent that despite the regenerative activity, partial hepatectomy accomplished the removal of significantly more functional reticuloendothelial

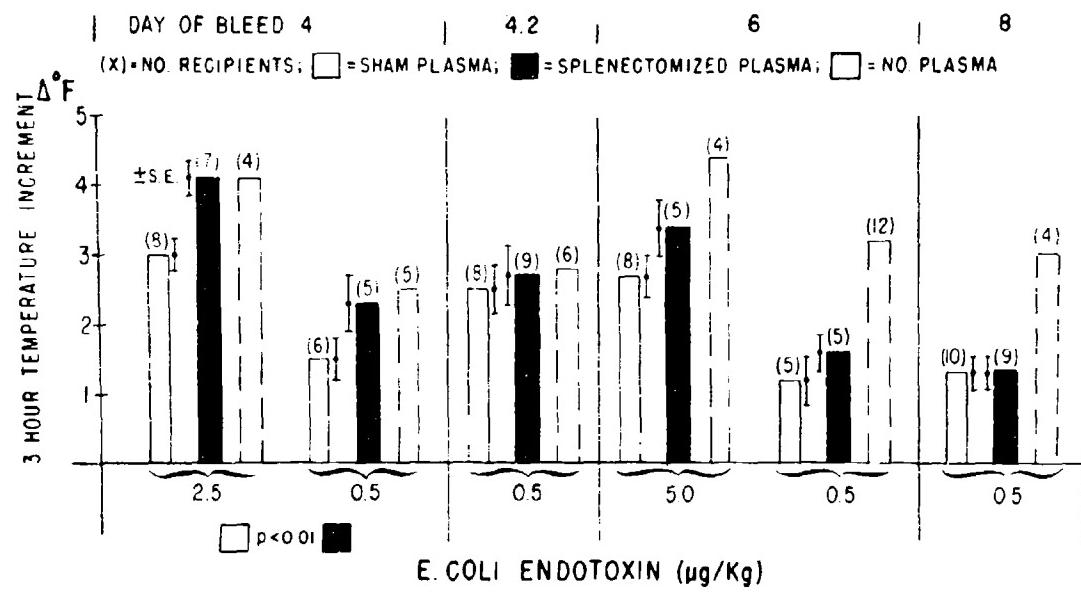


FIGURE 2. PASSIVE TRANSFER OF TOLERANCE FROM SPLENECTOMIZED DONORS.

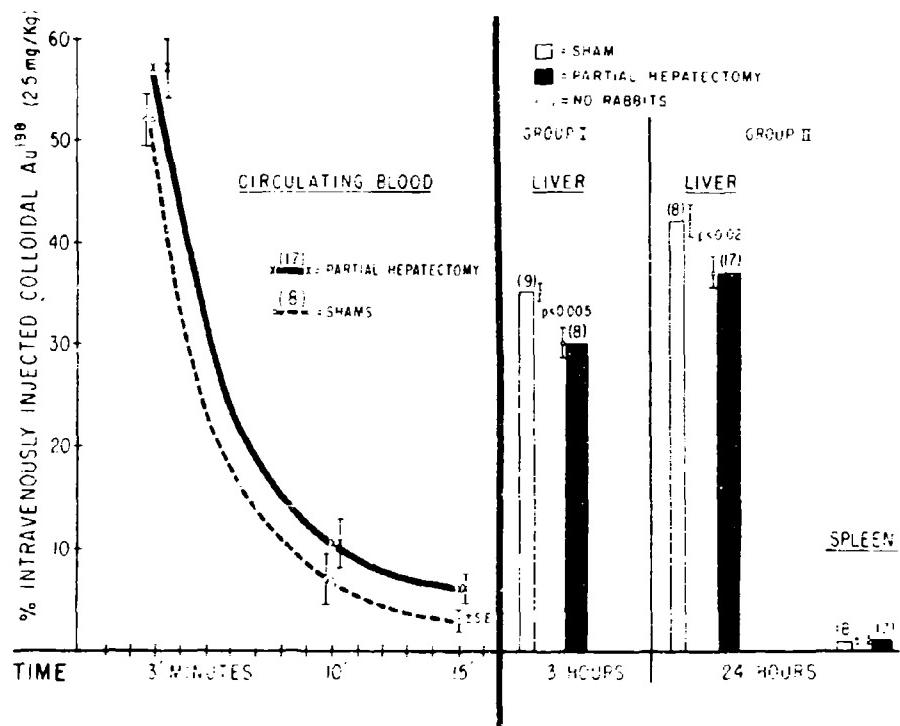


FIGURE 3. EFFECT OF PARTIAL HEPATECTOMY ON THE UPTAKE OF COLLOIDAL Au^{198} IN RABBITS.

tissue than did splenectomy. Moreover, since these clearance data were obtained after completion of the endotoxin tolerance studies more marked deficits of functional reticuloendothelium presumably existed during the period of endotoxin administration. Nevertheless, in contrast to the splenectomized host, the partially hepatectomized animals developed both pyrogenic tolerance and anti-"O" antibody titers to the E. coli endotoxin in a manner comparable to the sham-operated controls (Figure 4).

Effect of Splenectomy on Pyrogenic Tolerance and Anti-"O" Antibody to E. coli Endotoxin in Man.--The development of pyrogenic tolerance and of anti-"O" antibody following the IV administration of E. coli endotoxin was quantitated in groups of healthy volunteers with and without spleens. Because of severe initial toxic subjective reactions encountered in all subjects tested with a standard dose of E. coli endotoxin (0.01 µg/kg), and the increased toxic responses previously seen in man on day 2, re-testing was delayed until days 6, 8 and 11, i.e., only the late phase of tolerance was studied. The findings, depicted in Figure 5, can be summarized as follows: (1) pyrogenic and subjective toxic responses of control and splenectomized subjects were comparable following an initial IV injection of 0.01 µg/kg E. coli endotoxin; (2) pyrogenic tolerance to subsequent injection of the same dose developed in both splenectomized and control subjects, but tolerance acquisition was significantly retarded in the splenectomized group ($p < 0.02$ for days 6 and 8); (3) all control subjects receiving endotoxin developed progressive increases in anti-"O" antibody titers whereas no detectable antibody increments appeared in the splenectomized group and; (4) with respect to the tolerance retardation, the splenectomized subjects behaved comparably to control subjects receiving between 1/10 and 1/100 the standard doses of E. coli endotoxin. With respect to suppression of anti-"O" antibody production, the splenectomized subjects compared with controls receiving less than 1/100 the standard doses. Bactericidal antibody titers following the E. coli endotoxin administration paralleled those obtained by the bentonite flocculation technique.

SUMMARY

The initial pyrogenic responses of splenectomized rabbits and the tolerant responses at 24 and 48 hr after single IV injections of E. coli endotoxin, were identical to those of sham-operated controls. After 72 hr, however, splenectomized rabbits exhibited significant retardation of ability to acquire pyrogenic tolerance as well as impaired capacity to elaborate anti-E. coli endotoxin antibodies measured by anti-"O" antibody titers. In addition to the timing, evidence relating this late retardation of pyrogenic tolerance to impairment of antibody synthesis, rather than simply to loss of reticuloendothelial elements required to clear and inactivate the endotoxin was derived from (1) the parallel retardation in appearance of plasma factors capable of transferring pyrogenic tolerance and (2) the unaltered rates of development of pyrogenic tolerance

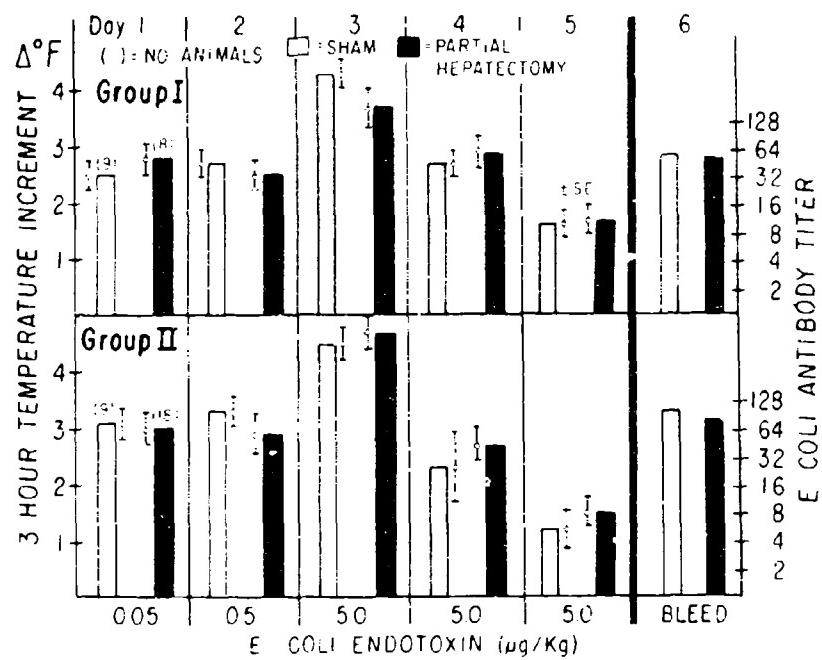


FIGURE 4. EFFECT OF PARTIAL HEPATECTOMY ON TOLERANCE AND BENTONITE-FLOCCULATING ANTIBODY AFTER DAILY ENDOTOXIN INJECTIONS.

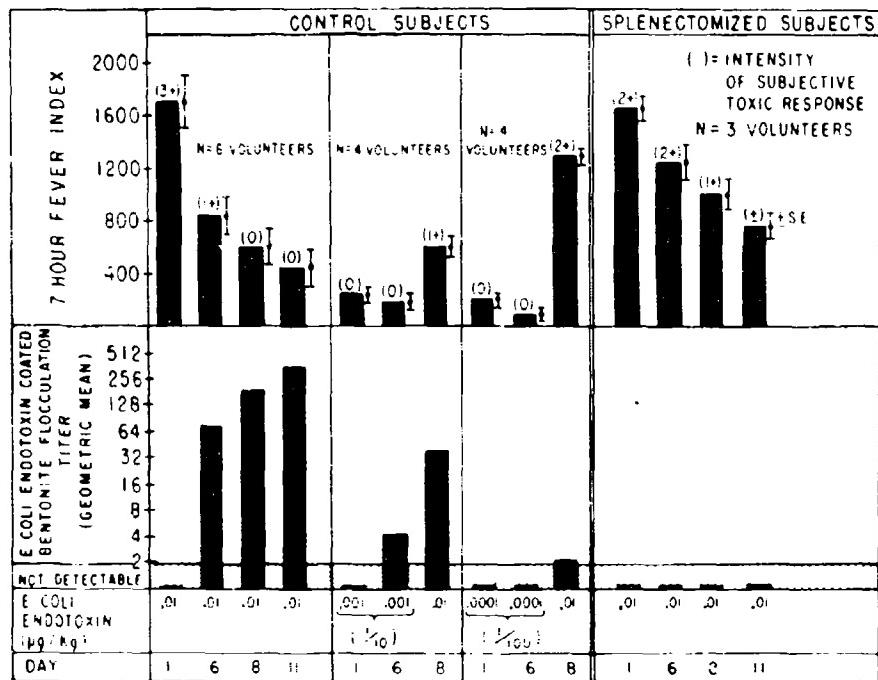


FIGURE 5. EFFECT OF SPLENECTOMY ON PYROGENIC TOLERANCE AND "O" ANTIBODY TITERS IN MAN.

(and of anti-"O" antibody synthesis) in partially hepatectomized rabbits lacking significantly more reticuloendothelium (as determined by uptake of colloidal Au¹⁹⁸), than the splenectomized host.

Similar to the rabbit, splenectomized man exhibited no enhanced initial pyrogenic or subjective toxic responsiveness to a standard dose of IV administered E. coli endotoxin, but upon retesting on days 6, 8, and 11 evidenced inability to elaborate circulating anti-"O" antibody and concomitant significant retardation of ability to acquire pyrogenic tolerance.

The above considerations are consistent with the hypothesis that pyrogenic tolerance to gram-negative bacterial endotoxins entails 2 distinct mechanisms: an early transient desensitization at the cellular level, and a subsequent elaboration of antibody which assist the RES in the clearance and destruction of this molecule.

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DISCUSSION

DR. KNIGHT: You showed very little clearance of radioactive gold by the spleen of hepatectomized animals. I presume these counts were made within a day of the injection of gold. It is my memory that when particulates are injected intravencously into mice, the clearance of any particle by the spleen is not as great the first day as it is at 4 or 5 days. There is a secondary mobilization coming from a very early and important clearance site. The spleen becomes a subsequent repository, having received material from lung and liver. Do you know anything about that point?

DR. GREISMAN: No. However, endotoxin in our studies would be cleared by the RES within a matter of hours, faster than the rates at which colloidal gold is cleared. Possible subsequent relocations of the delayed type

referred to above would not play a role in febrile responsiveness since this represents only the initial 5-7-hr reaction after endotoxin injection.

DR. CLUFF: You've referred to the immediate appearance of tolerance following the injection of endotoxins (either continuously or after a single injection) as being a phenomenon of desensitization? Do you imply that to be an allergic desensitization?

DR. GREISMAN: We wish we could answer that question. At least as a working hypothesis, we might consider an allergic type of desensitization and employ this for devising future experiments.

DR. WOOD: Dr. Greisman is to be congratulated for having stuck to this problem as long as he has. The going has been hard at times but he has come up with some beautiful data. I would like to ask how he would interpret the original Beeson observation in the light of his newer knowledge. In other words, how do you explain Beeson's thorotrast experiment?

DR. GREISMAN: Thank you very much for your kind remarks. The original experiments of Dr. Beeson showed conclusively that the injection of thorotrast or other RES blockading agents markedly enhanced the reactivity of the tolerant host to the action of the endotoxin molecule. However, this does not mean that tolerance (which represents the increment in resistance above that which the normal individual or normal animal possesses), is abolished by the injection of thorotrast or other colloidal agents. Rather, the host made tolerant to endotoxin actually retains tolerance. This has been shown in studies which demonstrate that the differences (i.e., increments) between "normal-blockaded" and "tolerant-blockaded" animals persist as before the block. The RES block has simply shifted the underlying setting, i.e., the baseline framework, of the reactivity of the normal and of the tolerant host to the endotoxin. Just how this is shifted, I am not certain. If Dr. Beeson, however had compared his RES blockaded-tolerant animals with comparably blockaded-normal animals, I think he would have agreed that their tolerance was not abolished.

STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN:
PROTEIN SYNTHESIS IN GUINEA PIG TISSUES AND PRIMARY HEART CELL CULTURES

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The factor which confers virulence to specific strains of Corynebacterium diphtheriae is the capacity to synthesize a protein exotoxin in the nasopharyngeal tissues of infected individuals. The protein toxin has been purified and crystallized and has been characterized extensively both chemically and immunologically.^{1-3/} These studies however have not contributed to an understanding of either the chemical moiety responsible for toxicity or to elucidation of the biochemical mode of action of diphtheria toxin. Presently, hypotheses concerning the biological action of the toxin are based primarily on information derived from tissue culture systems. Indeed, the experiments of Pappenheimer and Williams,^{4/} who utilized the developing Cecropia silkworm as the experimental animal, which pointed to the inhibition of tissue cytochrome systems as the mode of action of the toxin, had to be reevaluated in the light of tissue culture experiments which followed. Strauss and Hendee^{5/} made the initial significant observation that diphtheria toxin when incubated with HeLa cell cultures caused a virtual cessation of protein synthesis as measured by the incorporation of S³⁵-methionine into cell proteins. Inhibition of de novo synthesis moreover, occurred several hours before any evidence of the toxin's cytopathic effect became manifest. In view of the fact that the metabolism of HeLa cells is primarily glycolytic in nature, the complete inhibition of protein synthesis by the toxin could not be explained solely by an effect on the aerobic respiratory chain of which the cytochrome system is an integral part. Subsequent experiments by Collier and Pappenheimer,^{6/} who used cell-free protein synthesizing systems, provided unequivocal evidence that the toxin inhibited de novo protein synthesis mediated by both natural and synthetic messenger ribonucleic acid (RNA). They concluded that cells sensitive to diphtheria toxin are inhibited by a block in the protein synthesizing mechanisms after the activation of the amino acids and before their insertion on the messenger RNA template.

While the results obtained with the tissue culture systems present rather compelling evidence of the inhibition of protein synthesis as the mode of action of diphtheria toxin, it must be realized that tissue cultures do not represent a truly in vivo situation. In view of this, one is forced

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to ask questions relative to the biological action of the toxin: (1) How far can one extrapolate the information derived from the tissue culture and cell-free systems to the sensitive laboratory animal or human? (2) If inhibition of protein synthesis is the cardinal metabolic lesion induced by the toxin at the organismal level, which are the cell types and/or target organs involved? The experiments described here were designed to answer these important questions. A combined morphological and biochemical approach provided evidence that inhibition of protein synthesis is not a widespread metabolic effect of diphtheria toxin in the sensitive guinea pig. On the other hand, heart tissues were found to be uniquely sensitive in that protein synthesis was inhibited markedly as a result of diphtheria intoxication. The findings furthermore provide a biochemical rationale for the clinical syndrome exhibited by many patients with severe or fatal diphtheria infections.

The paper describes also the results of experiments in which primary heart cell cultures derived from embryonic guinea pigs and neonatal rats were exposed to diphtheria toxin and examined with respect to morphological changes and protein synthesis. The rationale for the experiments was to determine if species sensitivity or resistance was reflected under conditions where the cardiac tissues were removed from neural and endocrine influences, and before complete dedifferentiation had occurred. It was considered important that dedifferentiation be considered since established cell lines more often than not bear little resemblance to the tissues from which they originally were derived. The data presented corroborate the findings of the *in vivo* experiments in that species sensitivity or resistance to diphtheria toxin was exhibited by the primary heart cell cultures. They show further that it exerts a direct toxicity to cardiac tissues of a sensitive animal and that its effects on the intact heart are not secondary to neural or endocrine factors.

Diphtheria Toxin and Antitoxin.--Crystalline diphtheria toxin was obtained through the courtesy of Drs. M. F. Stevens and C. G. Pope (Wellcome Research Laboratories, England). The toxin when reconstituted contained 324 Lf units/mg; 1.0 Lf unit was equivalent to approximately 60 guinea pig median lethal doses (MLD). The purified crystalline toxin was used for all of the experiments. Purified diphtheria antitoxin was obtained through the courtesy of Dr. A. Pappenheimer, Jr. (Harvard Biological Laboratories). The potency of the antiserum was estimated to be 500 units/ml.

Animals.--Young Hartley strain guinea pigs (Simonsen Laboratories, White Bear Lake, Minn.) of either sex weighing 200-250 gm were used in all experiments. Young animals rather than adults were employed since protein turnover rates are more rapid. Randomly bred Swiss albino mice of mixed sexes (Hamilton Laboratories, Hamilton, Ohio) weighing 20-25 gm were used.

Heart Cell Cultures.--Pregnant guinea pigs (English strain) were supplied by a local animal dealer and Wistar rats were obtained from a breeding colony of the Department of Laboratory Animal Medicine, University

of Cincinnati College of Medicine. For each experiment 1 or 2 pregnant guinea pigs were used depending on the size of the litters. Pregnancy was terminated after 40-45 days of gestation. It was found that embryos obtained at this stage of development provided suitable heart tissues for establishing primary cell cultures. Full-term embryos apparently were too well developed for this purpose since they did not yield viable heart cell cultures. The litter of one rat was used for each experiment.

Primary cell cultures were established by a modification of the method described by Harary et al.^{7/} All manipulations were carried out under aseptic conditions. The embryos were removed quickly from the mother; heart tissues were minced and placed in Eagle's minimal essential medium (MEM),^{8/} containing 0.25% trypsin (Difco Laboratories, Detroit, Mich.). After 15 min of trypsinization at 37 C the tissues were centrifuged at low speed and the supernatant fluid discarded. The sedimented tissues were resuspended and trypsinized for an additional 45-60 min. The heart cells were then collected by centrifugation and suspended in MEM containing the appropriate serum. Guinea pig heart cells were collected in MEM + guinea pig serum (final concentration, 8%) and rat heart cells, in MEM + calf serum (final concentration, 10%). In both cases the cells were suspended to an optical density of 0.06 in the appropriate tissue culture fluid. Monolayers of the heart cells were obtained by growth either on cover slips (3/60-mm Petri dish) or on the surface of 60-mm plastic dishes (Falcon Plastics, B-D Laboratories, New York, N. Y.). Tissue culture fluids were changed every 48 hr during incubation at 37 C under an atmosphere of 95% air and 5% CO₂. Confluence monolayers were achieved 5-7 days after initiating the cultures. Beating centers of heart cells were seen routinely in the rat heart cell cultures beginning approximately 2 days after the cultures were established. Guinea pig heart cells which exhibited rhythmic contractions were seen occasionally but not in all cultures. Two distinct morphological types of cells could be identified in the cultures; one was identified as endothelioid, derived from the capillaries of the heart muscle, and the second, myocardial heart muscle. The endothelioid cell could be identified by its clear cytoplasm, large nucleus containing multiple nucleoli, and absence of myofibrils; a typical myocardial muscle cell was characterized by a dense cytoplasm containing numerous sarcosomes, a well-defined small nucleus containing one nucleolus and surrounded by a double membrane-like structure (Figure 1A). Typical myofibrils of the heart cells could be visualized, although not with uniform success by silver nitrate staining (Figure 1B).

Protein Turnover in Animal Tissues.--Guinea pigs were fasted for 24 hr prior to the start of each experiment. Like the tissue cultures, the animals were divided into 3 groups: (1) diphtheria toxin treated, (2) toxin-anti-toxin treated, and (3) controls. The diphtheria toxin group was injected intramuscularly (IM) with 20 MLD of the crystalline toxin. This quantity of toxin was sufficient to cause death of the animals 24-30 hr after injection. The toxin-antitoxin animals were injected with 250 units of antitoxin intraperitoneally (IP) 30 min prior to the toxin (20 MLD IM). The control animals were injected with saline. Approximately 20 hr after the first injection,



FIGURE I. PRIMARY HEART CELL CULTURE ESTABLISHED
FROM THE HEART TISSUES OF NEONATAL RATS.
TYPICAL HEART CELLS-1; EPITHELIOID CELL-2.
(LEFT: PHASE CONTRAST,X 512; RIGHT: SILVER NITRATE, X 1280).

at which time the diphtheria toxin injected animals showed visible signs of illness, the animals were given IP 1.5 mc of H³-leucine. The labeled amino acid was administered either in one dose or in 2 equal doses 1 hr apart. After a 2-hr exchange period the animals were sacrificed by cardiac perfusion with a buffered formalin 3% sucrose fixative. The desired tissues were then excised rapidly and placed in the fixative for several days, or frozen at -70 C for storage until analyzed. The fixed tissues were used for autoradiography and the frozen tissues extracted for protein determinations and radioactivity measurements. Occasionally fixed tissues were also used for protein extraction and radioactivity determinations.

After the first guinea pig experiment with 6 animals, we became aware of several unpublished experiments which established that conditioning of the guinea pigs to manipulation and injection may affect the rate and extent of incorporation of labeled amino acids into tissue proteins.⁹ It was shown that animals reared in isolation demonstrated a greater incorporation of labeled amino acids into brain tissue than did animals raised in an "enriched" environment; i.e., together with other guinea pigs, and adapted to handling and injection procedures. Since this situation could conceivably present another experimental variable, all animals except those used in the first experiment were conditioned for 2 weeks prior to the start of any experiment by housing them in groups of 4-6 and also by adapting them to IP injections every other day with 1.0 ml of physiological saline.

Mice were treated like the guinea pigs except that the quantity of H³-leucine injected was reduced to 0.3 mc. In view of the fact that mice are comparatively resistant to diphtheria toxin, they were injected with 100 MLD of diphtheria toxin. All mice were refractory and survived.

Incorporation of Tritiated Leucine into Heart Cell Proteins.--The primary heart cell monolayers were divided into 3 groups for each experiment: (1) incubated with diphtheria toxin; (2) incubated with a toxin-antitoxin mixture; and (3) incubated with tissue culture medium (controls). The heart cell cultures were exposed to concentrations of toxin between 10 and 100 MLD/ml of fluid for various lengths of time, 3-4 hr. After incubation with the toxin the tissue culture fluids were replaced with medium containing H³-leucine (specific activity 500 mc/mM) at a concentration of 5 μ c/ml. After an exchange period of 3-4 hr the cell cultures were rinsed thoroughly with MEM and the proteins precipitated with cold 0.5 M perchloric acid. The precipitates were washed twice in this acid before protein assays and radioactivity determinations.

Protein Determinations.--Animal tissues were homogenized in distilled water and precipitated with cold 5% trichloroacetic acid (TCA). The precipitates were washed 2 times with cold TCA and then extracted successively with hot (60 C) 50%, 95% and absolute alcohols; ether and absolute alcohol (1:1); and finally 3 times with ether. The dried residue was then analyzed for protein content by the method of Lowry et al.¹⁰ as modified by Oyama and Eagle.¹¹

Radioactivity Measurements.--Tissue culture samples were digested in minimum volumes of 0.1 N NaOH. The dried extracted tissue residues were digested in minimum volumes of 2.0 N NaOH. Minimum volumes were employed since large aqueous samples could not be added to the liquid scintillation mixture employed without separation into a 2-phase system. All samples were prepared for liquid scintillation counting in the DTN (dioxane-toluene-naphthalene) thioxotropic gel (Cab-O-Sel, Packard Instrument Co., Downers Grove, Ill.) mixture described by Engel.^{12/} The samples were counted in a model 574 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.). Radioactivity values were expressed as counts per minute per mg protein (cpm/mg).

Histological Procedures.--Fixed animal tissues were embedded in "Paraplast" (Aloe Scientific, St. Louis, Mo.) sectioned at 5-6 μ , and stained directly. In cases where staining of tissue autoradiograms was done, the sections were stained through the emulsion after photographic development. In all cases, the stain used was Harris' hematoxylin and Eosin B.^{13/}

The heart cell cultures were stained either with a Giemsa stain or a modification of the Rio-Hortega silver stain.^{14/} Unstained preparations were examined by phase microscopy with a Zeiss research photomicroscope.

Radioautography.--Animal tissue sections were deparaffinized with xylol and hydrated through graded alcohols and distilled water. The processes of fixation before sectioning and hydration after sectioning removed all of the unbound H³-leucine. Therefore, all of the residual radioactivity was associated with cell proteins.

The tissue sections were prepared for radioautography in a darkroom equipped with safelight. The nuclear emulsion (NTB-3, Eastman Kodak Co., Rochester, N. Y.) was liquified in a waterbath adjusted to 42 C and stirred gently for 1 hr to release air bubbles trapped within the fluid. The slides were prewarmed to 42 C and dipped individually in the emulsion for 2 sec. Excess emulsion was drained and wiped off the back with a soft cloth. Slides were allowed to dry in a horizontal position in an enclosed chamber through which air circulated freely. The emulsion-coated slides then were placed in light-tight slide boxes containing dessicant and stored at 4 C. After suitable exposure periods (5-16 weeks) the autoradiograms were developed in D-19 developer (Eastman Kodak Co.) equilibrated to 20 C. The entire radioautographic procedure was standardized rigidly so that differences observed in the autoradiographic images could not be ascribed to variations in the experimental manipulations.

Microdensitometry.--Until recently, one of the major objections to radioautography was that it did not lend itself readily to quantitative evaluation. In spite of this, autoradiographic data of a quantitative nature could be obtained if one resorted to the tedious chore of counting exposed silver grains per cell or unit area. A major advance in the quantitation was made by Altman^{15,16/} who showed that radioactivity

could be correlated with grain density by microdensitometric analysis. He established that density was linearly related to the number of exposed silver grains over any circumscribed area measured. The method facilitates the evaluation of numerous autoradiograms by substituting optical density values for silver grain counts.

Comparison of Protein Synthesis in Guinea Pigs and Mice.--It was assumed that the resistance of mice should be reflected in minimal inhibition of leucine incorporation into tissue proteins. Experiments were designed to compare protein synthesis in the tissues of guinea pigs and mice after challenge with diphtheria toxin. The results presented were compiled from the data of 2 separate experiments involving 10 guinea pigs and 4 mice. The data derived from densitometric readings of the tissue autoradiograms (Table I) and the radioactivity of the tissue proteins (Table II) show clearly that all of the guinea pig tissues examined except the pancreas and heart were unaffected by the toxin. A slight inhibition of leucine incorporation into serum proteins was observed. None of the mouse tissues showed a reduction in the extent of leucine incorporated. It should be noted that diphtheria toxin had no inhibitory effect on protein synthesis of mouse tissues in spite of the fact that mice were challenged with a 5-fold greater quantity of toxin (100 MLD) than were the sensitive guinea pigs.

Only 2 guinea pig tissues were inhibited by the toxin; the heart appeared to be involved to a greater extent and in a more consistent manner than the pancreas. The composite data show that the mean value of inhibition of leucine incorporation into heart protein was 44% by microdensitometric analysis and 55% by direct radioactivity measurements. The pancreas on the other hand was inhibited to a lesser degree, 27 and 44% by the respective methods of measurement. We considered these differences to be important. While unequivocal evidence is lacking, the metabolic lesion induced in heart tissues appears to be more significant as a primary *in vivo* effect of diphtheria toxin than the pancreatic lesion. The autoradiographic evidence also suggests that the inhibition manifested in the heart tissue is generalized and not restricted to specific anatomic regions. This is shown by the paucity of exposed silver grains over the entire heart autoradiogram (Figure 2, left). In contrast on the right is a section from a normal guinea pig showing uniform labeling throughout the section.

Inhibition of Protein Synthesis in Guinea Pig Heart Tissue.--Since diphtheria toxin appeared to exert its most significant effects on heart tissue, another experiment was designed in which the period of incorporation of H^3 -leucine was extended for 2-6 hr. The rationale for the prolonged exchange period was that all muscle tissues, including the heart, have relatively low rates of protein turnover,¹⁷ consequently an extended incubation period with the labeled amino acid might accentuate the inhibition exerted by the toxin. That this proved to be the case is shown by the data in Table III. It can be seen that the inhibition of leucine incorporation into heart proteins counting of extracted proteins was 73%. In view of the greater inhibition observed with the extended exchange

TABLE I. EFFECT OF DIPHTHERIA INTOXICATION ON THE INCORPORATION OF H^3 -LEUCINE INTO GUINEA PIG TISSUES AS MEASURED BY SILVER GRAIN DENSITIES OF AUTORADIOGRAMS

TISSUE	% REDUCTION IN GRAIN DENSITY ^a /	
	Diphtheria toxin	Toxin-antitoxin
Brain (neurons)	0	-
Small intestine (villi)	0	-
Spleen (germinal centers)	0	-
Kidney	0	-
Adrenal	0	-
Liver	0	0
Pancreas (acini)	27	4
Heart	44	0
Right ventricle	76	12
Left ventricle	72	0
Right atrium	44	0

a. Composite data obtained by microdensitometric evaluation of tissue sections from 10 guinea pigs. Values calculated on basis of optical density measurements of tissue autoradiograms as compared with autoradiograms prepared from control guinea pigs.

TABLE II. $\text{H}^3\text{-LEUCINE INCORPORATION INTO TISSUE PROTEIN OF NORMAL AND DIPHTHERIA TOXIN TREATED GUINEA PIGS AND MICE}^{\text{a}/}$

TISSUE	RADIOACTIVITY (cmp $\times 10^{-3}$ /mg protein) ^{b/}				
	Guinea pigs			Mice	
	Normal	Toxin	% Inhibition	Normal	Toxin
Liver	18.8	19.0	ns ^{c/}	11.0	19.6
Spleen	17.2	16.5	ns	40.7	47.5
Small intestine	27.7	34.9	ns	46.3	77.8
Adrenal	23.0	24.7	ns	4.3	4.1
Lung	7.8	7.0	ns	14.4	18.3
Brain	5.6	6.0	ns	8.5	9.1
Diaphragm	16.9	16.3	ns	19.5	14.5
Serum	20.8	16.3	21.0	--	--
Pancreas	111.9	63.2	44.5	60.0	73.6
Heart	7.7	3.5	55.0	8.5	9.1

a. Composite data from 10 guinea pigs or 4 mice.

b. Average radioactivity values of samples counted in duplicate or triplicate.

c. ns = No statistical difference.

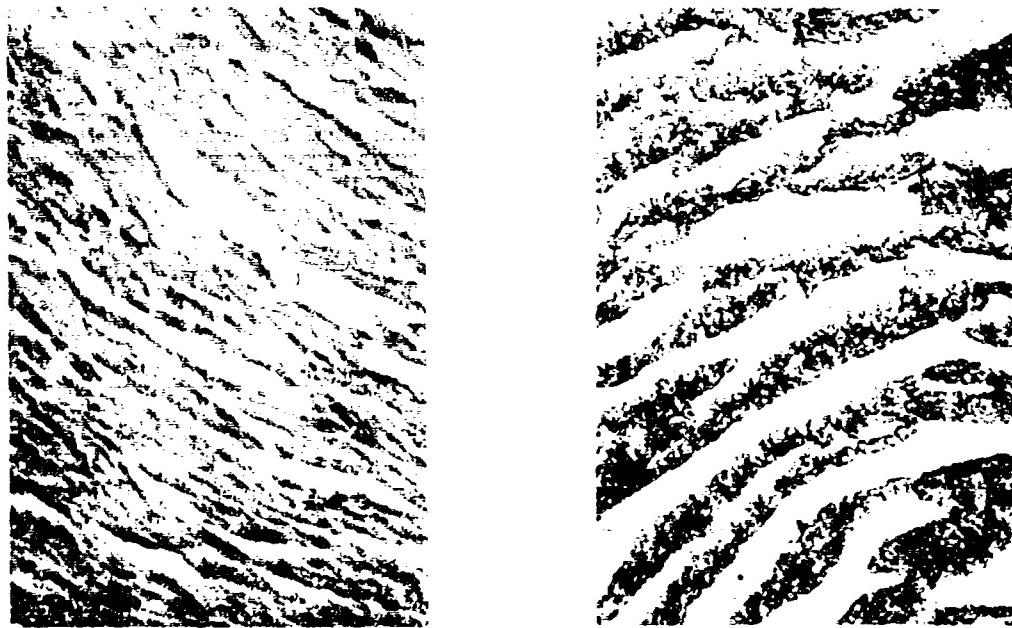


FIGURE 2. AUTORADIOGRAMS OF UNSTAINED HEART TISSUE
SECTIONS FROM GUINEA PIGS INJECTED WITH
1.5 mc TRITIATED LEUCINE 2 HR BEFORE SACRIFICE.
(LEFT: DIPHTHERIA-TOXIN INJECTED; RIGHT: NORMAL CONTROL, X 205).

TABLE III. INHIBITION BY DIPHTHERIA TOXIN OF PROTEIN SYNTHESIS IN GUINEA PIG HEART TISSUES AS MEASURED BY INCORPORATION OF H^3 -LEUCINE^a/

TREATMENT	RADIOACTIVITY (cpm $\times 10^{-3}$ /mg protein)	% INHIBITION
Normal (control)	7.86	
Toxin	2.18	73
Toxin-antitoxin	7.42	0

a. Guinea pigs given 1.5 mc H^3 -leucine IP 6 hr prior to sacrifice.

period it is reasonable to assume that the closer the animal is to death, the higher would be the inhibition of protein synthesis demonstrable. In our experiment the animals had become moribund when they were sacrificed by cardiac perfusion.

It was also considered important to look at the heart autoradiograms with respect to the densities of different anatomic regions. Although inhibition of leucine incorporation was apparent throughout the entire heart sections, some variation in densities was noted (Figure 3); e.g., the ventricles seemed to be inhibited to a greater degree than the atrium. At this juncture it is difficult to attach any physiological significance to this observation but it may prove to be important if the toxin is found to inhibit specific proteins or enzymes of the heart.

Effect of Diphtheria Toxin on Rat Heart Cells.--Rat heart cell cultures were refractory to the action of diphtheria toxin. The typical rhythmically beating cells were unaffected and morphology was unchanged even after a prolonged incubation period of 42 hr with concentrations of the toxin as high as 100 MLD/ml. In addition protein synthesis as measured by the incorporation of H^3 -leucine remained essentially normal in the presence of toxin (Table IV). Ten, 25 and 100 MLD/ml had no significant effects on protein synthesis. It was seen that toxin-antitoxin mixtures using unpurified rabbit antiserum caused an inhibition of protein synthesis but the inhibition was ablated when the crude antitoxin was replaced by a purified rabbit globulin preparation. Presumably the rabbit antisera contained materials which were inhibitory for the rat heart cells in culture. Although tissue cultures usually require serum for optimum growth the cells can at times be extremely sensitive to undefined toxic factors present in some batches of either homologous or heterologous sera. These data suggest that the species resistance of the rat to diphtheria toxin is reflected by the resistance of the primary heart cells to the action of the toxin.

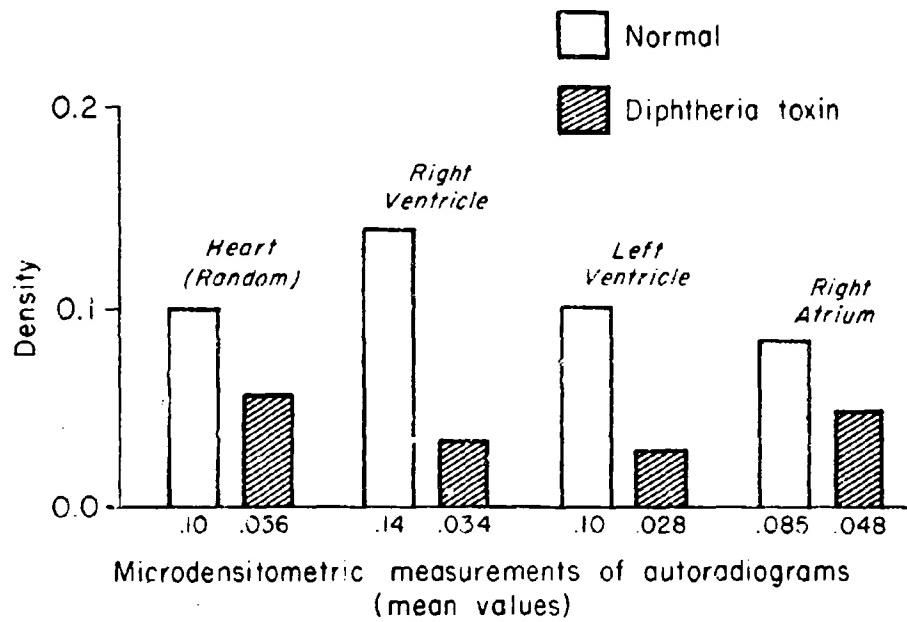


FIGURE 3. OPTICAL DENSITY VALUES OF UNSTAINED GUINEA PIG HEART TISSUE AUTORADIOGRAMS PREPARED FROM NORMAL AND DIPHTHERIA-TOXIN TREATED ANIMALS.

TABLE IV. INCORPORATION OF H^3 -LEUCINE INTO PROTEINS OF PRIMARY RAT HEART CELL CULTURES (NEONATAL RAT)^a

TOXIN MLD/ml	TIME INCUBATED WITH TOXIN (hr)	RADIOACTIVITY (cpm $\times 10^{-3}$ /mg protein)		
		Normal	Toxin	Toxin-Antitoxin
10	18	30.5	27.5	22.9 ^b /
10	42	30.5	31.7	--
25	18	24.0	22.1	14.1 ^b /
25	42	24.0	19.3	--
100	6	178.8	183.2	178.6 ^c /
100	18	178.8	174.5	182.8 ^c /

Composite data of 3 experiments.

- a. Cell monolayers incubated with toxin for specific number of hours and tissue culture fluids (MEM) then replaced with fluids containing 5 μ c/ml of H^3 -leucine for 4 hr.
- b. Whole hyperimmune rabbit serum used as antitoxin.
- c. Purified γ -globulin fraction of hyperimmune rabbit serum used as antitoxin.

Effect of Diphtheria Toxin on Guinea Pig Heart Cells.--In contrast to the rat heart cell cultures, guinea pig heart cells in primary cultures were found to be extremely sensitive. Guinea pig heart cells incubated with 25 MLD/ml of diphtheria toxin incorporated leucine to a much lesser extent than did the untreated cells. Incubation with the toxin for 3-48 hr resulted in inhibition of protein synthesis between 37 and 53% (Table V). Increasing the concentration of toxin to 100 MLD/ml did not result in inhibition of protein synthesis greater than that observed with the smaller amounts of toxin (Table VI). These results are in agreement with the observations made in vivo where protein synthesis in cardiac tissues was inhibited equally in animals injected IM either with 20 or 1,000 MLD. It should be noted also that purified antitoxin completely reversed the inhibition.

The cytopathic effect of diphtheria toxin was also manifested with the primary guinea pig heart cell cultures. The cells exposed to the toxin exhibited increased granularity and vacuolization with time; after 72 hr

TABLE V. INCORPORATION OF H^3 -LEUCINE INTO PROTEINS OF PRIMARY GUINEA PIG HEART CELL CULTURES (EMBRYONIC GUINEA PIG)^{a/}

TIME INCUBATED WITH TOXIN (hr)	RADIOACTIVITY (cpm $\times 10^{-3}$ /mg protein)		
	Normal	Toxin	% Inhibition
3	171.8	108.2	37
6	145.9	79.2	46
24	135.5	64.4	53

Composite data of 4 experiments.

- a. Monolayers incubated with H^3 -leucine (5 μ c/ml) 3 hr after incubation with 25 MLD/ml diphtheria toxin for specified number of hours.

TABLE VI. EFFECT OF DIPHTHERIA TOXIN ON PROTEIN SYNTHESIS BY GUINEA PIG EMBRYO PRIMARY HEART CELL CULTURES^{a/}

TREATMENT	RADIOACTIVITY (cpm $\times 10^{-3}$ /mg protein)			
	3 hr toxin	% inhibition	6 hr toxin	% inhibition
Normal	195.0	--	195.0	--
Toxin (100 MLD/ml)	89.7	54.0	97.8	50.0
Toxin-Antitoxin ^{b/}	--	--	188.4	3.4
Antitoxin ^{b/}	--	--	181.3	7.1

- a. Cell cultures incubated with toxin and/or antitoxin before incubation with H^3 -leucine (5 μ c/ml) for 3 hr.
- b. Purified γ -globulin function of rabbit hyperimmune serum used as antitoxin.

the monolayers were destroyed (Figure 4). Thus the primary guinea pig heart cells reflected the typical species sensitivity to diphtheria toxin with respect to both inhibition of protein synthesis and direct cytotoxicity.

Densitometric analysis of tissue autoradiograms and liquid scintillation counting of tissue proteins have provided evidence that inhibition of protein synthesis is not a generalized metabolic effect of diphtheria toxin in an *in vivo* situation. The only guinea pig tissues found to be inhibited were the pancreas and the heart, with the latter being affected more extensively and consistently. This inhibition of protein synthesis was not seen in heart and pancreas of the resistant mouse. The specificity of inhibition was also shown by the reversal of the effect when guinea pigs were protected with diphtheria antitoxin. When the exchange period was increased for 2-6 hr, the inhibition of leucine incorporation into guinea pig heart tissues was more apparent (73%).

The data become extremely provocative when one attempts to correlate them with a hypothetical site and mode of action of diphtheria toxin *in vivo*. It is clear that if inhibition of protein synthesis is the prime metabolic lesion induced by the toxin, its action *in vivo* is restricted to a limited number of tissue proteins. This specificity is in no way indicated by the results obtained with HeLa cells or other tissue cultures where inhibition is virtually an all-or-none phenomenon. It is difficult on the basis of the experimental data to assess the significance of the inhibition of protein synthesis noted in the guinea pig pancreas. It can be speculated, however, that a reduction in the level of pancreatic enzymes secreted, even if substantial, would not constitute a pathological situation which by itself would result in death of guinea pigs within hours. On the other hand, the same metabolic lesion in heart tissues is of much greater significance since such a disturbance would ultimately affect heart function and consequently represents a life-threatening situation. These considerations, coupled with the data, suggest that a plausible explanation of the biochemical mode of action of diphtheria toxin in the sensitive mammalian host is the specific inhibition of heart protein synthesis.

Correlation of clinical evidence with the data is also pertinent. It has long been common knowledge that cases of human diphtheria which terminate fatally are often associated with cardiac failure. In addition, many of the deaths occurred during convalescence after the infection had subsided or reached a subclinical level.^{18/} Examination of heart tissues after death has also indicated cardiac injury in many cases although the pathological lesions found have been varied and inconsistent. Pathological changes in both the heart parenchyma and interstitial tissues have been described, with the most pronounced effects being noted in those patients who survived for a considerable time, or those who had suffered the extremely severe "bull neck" form of diphtheria. This observation may be significant in the present investigation since no pathological changes could be detected in the heart tissues of guinea pigs which succumbed to the toxin. It may be that the relatively short survival time of the animals after challenge was not sufficient to allow a morphological expression of the biochemical lesion.

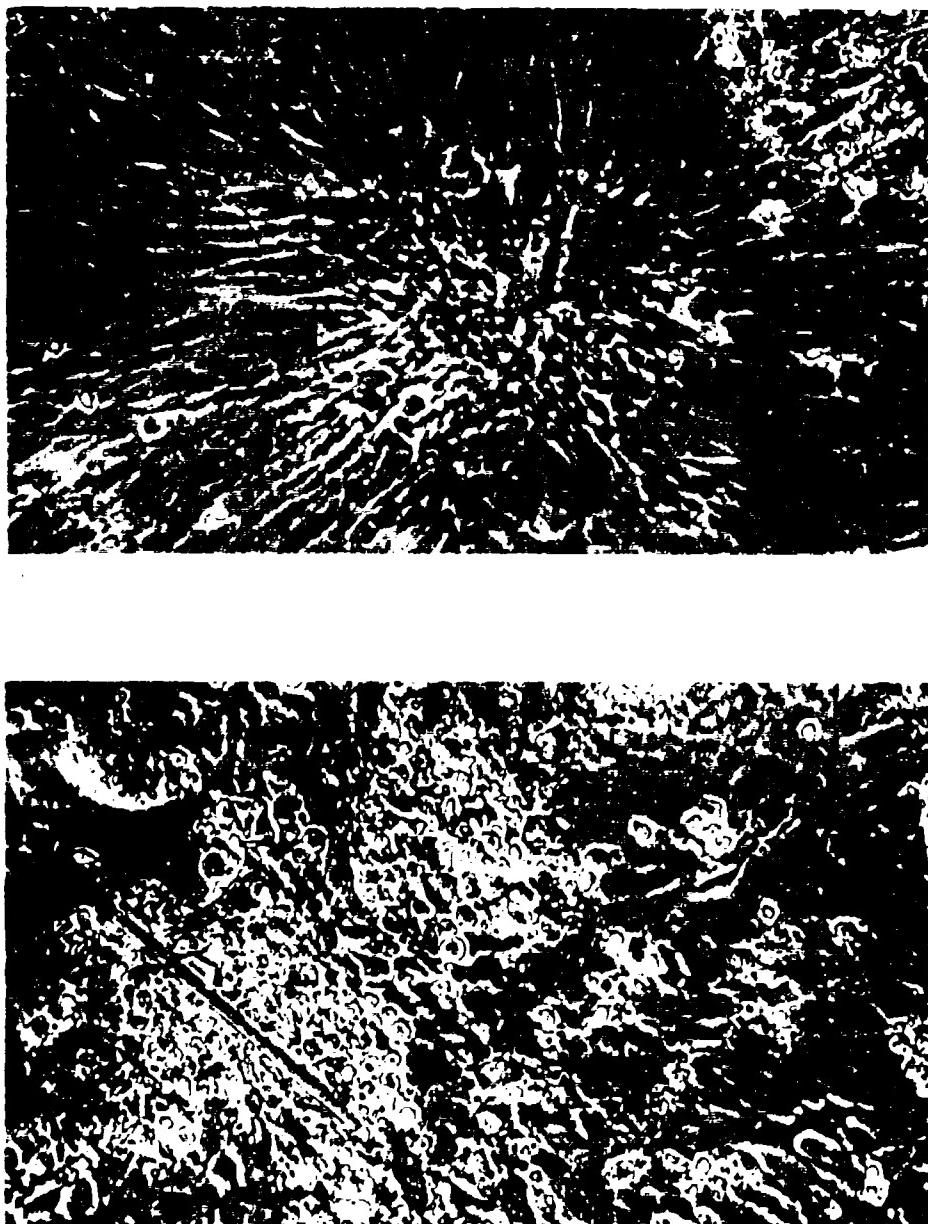


FIGURE 4. PRIMARY EMBRYONIC GUINEA PIG HEART CELL CULTURE. (ABOVE: NORMAL, 7-DAY, X 128; BELOW: INCUBATED WITH DIPHTHERIA TOXIN, 48 HR, X 320. PHASE CONTRAST).

At this point it would be premature to conclude that the significant primary biochemical lesion induced by this toxin is the one described here. It may be that other tissues not examined are affected or that inhibition of protein synthesis per se is not directly responsible for the pathophysiology of diphtheria. On the other hand, the specific inhibition of protein synthesis noted in the heart tissue of the exquisitely sensitive guinea pig warrants further study. The autoradiographic data suggest that although inhibition of protein synthesis is manifested throughout the entire heart tissues, certain anatomic regions are more involved than others. It may be that the action of the toxin is limited to specific structural proteins or alternatively to specific enzyme systems of the heart. In order to investigate these possibilities, experiments with primary cultures of neonatal and embryonic heart tissues obtained from resistant and sensitive animal species were conducted.

The apparent specificity of cardiac tissues as the site of action of diphtheria toxin was substantiated. Heart cells derived from the embryonic guinea pig, in primary culture were found to be extremely sensitive. The inhibition of protein synthesis and the cytopathic effects noted in the absence of neural or endocrine stimuli show that diphtheria toxin exerts a direct toxicity on these muscle cells. The direct effect on heart cells in culture suggests further that the action of the toxin on cardiac tissues in the intact guinea pig is not due to secondary or terminal effects. Finally, it can be concluded that the deleterious effects on the heart are manifested only in those mammalian species which are not endowed with a natural resistance to the protein toxin.

Are the results obtained with the intact guinea pig and the primary heart cell cultures compatible with the findings using established tissue cultures and cell-free systems? The current hypothesis advanced by Collier¹⁹/ and Goor²⁰/ is that the toxin acts by binding a transferase through nicotinine adenine dinucleotide (NAD) and thereby prevents the formation of the polypeptide chain. Since transferase enzymes are presumably present in all types of mammalian cells²¹/ one would expect that inhibition of protein synthesis would be apparent in all tissues and not restricted to the heart as our experiments suggest. However, it may be that the toxin binds specifically to cardiac tissues and is localized in high concentration at that site because of a peculiar tissue affinity for the protein toxin. Although this is an unlikely possibility we are now testing it experimentally by means of fluorescent antibody methods. If tissue affinity for the toxin proves not to be the answer, it will be necessary to seek an explanation of cardiac specificity by examining in detail the biochemical machinery of the heart tissues. It is an intriguing situation since heart tissues are composed almost exclusively of structural proteins which are replenished at a relatively slow rate. One would expect a priori that tissues such as the small intestine which turn over at a fast rate would be the likely sites at which the toxin exerts its action. No such inhibition however could be detected in our in vivo experiments. If it were possible to detect a unique feature in the structural components or the enzymatic makeup of the

protein synthesizing machinery of cardiac tissues then it may be possible to explain in molecular terms why diphtheria intoxication appears to manifest itself as a heart disease.

SUMMARY

The effect of crystalline diphtheria toxin on protein synthesis *in vivo* was evaluated in guinea pigs and mice. By 2 independent methods of analysis it was established that inhibition of protein synthesis was not a widespread metabolic effect of diphtheria toxin. In the sensitive guinea pig, only the heart and pancreas showed any demonstrable reduction in the quantity of H^3 -leucine incorporated into protein. In the resistant mouse no such inhibition was noted. It was suggested that inhibition of protein synthesis in heart tissues could provide a biochemical rationale for the site and mode of action of the toxin in the sensitive mammalian host.

Primary heart cell cultures of embryonic guinea pigs and the neonatal rat were established and incubated with the toxin. Rat heart cell cultures were refractory, as would be expected in this resistant animal. Guinea pig heart cells were extremely sensitive, 50% inhibition after incubation with the toxin for 3 hr. Monolayers were destroyed when incubated with the toxin for 2-3 days.

The results show that the heart cells reflect species resistance of sensitivity to diphtheria toxin in the absence of neural or endocrine influences and suggest that there is direct toxicity to the cardiac tissues. Biochemically it is not yet possible to explain this specificity.

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DISCUSSION

DR. KLAINER: A fruitful place to look for diphtheria toxin would be in the nerve, inasmuch as two types of neuritis occur in diphtheria, an early and a late. Another point is that when people die of diphtheria or diphtheritic myocarditis, those people who die late have big flabby hearts, while those who die early (in a manner similar to your guinea pigs who get toxin IM) die from arrhythmia. Rather than just biopsying or testing the right and left ventricles, you might look at the nodal system or the bundle.

DR. BONVENTRE: Those are very good suggestions, but I might point out that in order for nerve involvement to take place in an experimental system one must resort to treating the animal with a suitable quantity of anti-toxin to protect him partially. In the chronic situation there occurs the so-called Wallarian degeneration of nerve fibers. I must admit that the histologic study of cardiac tissues in our experiments were completely negative for pathological changes.

DR. GRAY: In some studies on the mechanism of SEB effect, in the target organ, the lung, there occurred a very definite inhibition of oxidative metabolism. The mechanism of this inhibition involved the prevention of reoxidation of reduced NAD, thereby making NAD unobtainable to the tissue. This may be in accord with the postulate of Collier and others on how this may act.

DR. BONVENTRE: Yes, but their finding was that the toxin will not act in the absence of NAD.

DR. GRAY: But does the toxin have an effect on the concentration of NAD?

DR. BONVENTRE: Well, this would be a very difficult thing to establish in vivo. I don't know how far we can extrapolate this hypothesis to the whole animal situation. Dr. Pappenheimer in his most recent papers admits that the in vivo situation is infinitely more complex than the system he

and his students have employed, and that their results with cell-free protein synthesizing systems cannot explain the nature of the in vivo events during diphtheria intoxication.

DR. GRAY: But if any of these toxins prevent or decrease oxidative metabolism then protein synthesis should also be affected.

DR. BONVENTRE: That would depend on the metabolic pathways utilized by the cells being studied. Cell types which are primarily anaerobic would probably not be inhibited significantly.

DR. CLUFF: You indicated that the toxin has similar effects on HeLa cells in tissue culture. And it also has effect on cardiac cells following inoculation of the experimental animal. Did you demonstrate the same effect upon myocardial tissue culture?

DR. BONVENTRE: Yes, in vitro.

DR. CLUFF: You have demonstrated the specificity from myocardial tissue in vivo, and yet toxin appears to have an effect upon the HeLa cell, which if I am not mistaken is a cervical cell carcinoma. Have you utilized any other cell lines in tissue culture?

DR. BONVENTRE: The original papers published by Lennox and Kaplan and later Gabliks reported on an entire spectrum of cells. Both groups found that many of these established cell lines were sensitive, but one was resistant; no pattern emerged.

DR. CLUFF: So the specificity is primarily demonstrable following inoculation in the intact animal.

DR. BONVENTRE: Correct.

THE LOCALIZATION OF STAPHYLOCOCCAL ENTEROTOXIN B

Captain Sigurd J. Normann, MC*

Staphylococcal enterotoxin B (SEB) is an exotoxin elaborated by Staphylococcus aureus which can produce the symptoms of acute staphylococcal food poisoning in experimental animals.^{1,2/} This toxin which was originally isolated and purified by Schantz and co-workers,^{3,4/} can also produce lethargy, fever, shock and death in monkeys and rabbits when administered intravenously (IV) in sufficient dosage.^{5,6/} Since it is possible that certain symptoms of staphylococcal disease may be related to enterotoxemia, an appreciation of how this toxin causes illness may aid in the eventual understanding and management of staphylococcal infections.

Although SEB can produce death in experimental animals, the pathology of the disease shows little to explain the cause of death. The principal pathologic lesion so far described appears to be pulmonary interstitial edema.^{7/} However, since pulmonary edema is not consistently seen in all animals, nor is it generally sufficient to account for death, other methods besides morbid pathology appear necessary to define the initial sites of toxin localization and possible sites of action.

One approach to the study of toxin distribution in the intact animal is to label the toxin with either a radioactive compound or a fluorescent reagent; a somewhat different approach is to identify the toxin in tissues by fluorescent antibody methods. The labeling of the toxin with I¹³¹ has already been reported; Crawley and co-workers^{8/} concluded that the lung was the major site of toxin localization. However, subsequent studies have shown that IV injected I¹³¹-labeled toxin is rapidly removed from the blood and that such rapid removal is markedly diminished by bilateral renal artery ligation.^{8,9/} The latter observation together with the fact that considerable radioactivity accumulates in the kidney^{10/} suggests that the kidney rather than the lung is the important site for the early removal of the toxin. Two questions can now be raised: (a) if the toxin is removed by the kidney, what is the mechanism of renal clearance, and (b) what are the principal cellular sites of toxin localization in the kidney?

It is the purpose of the present communication to describe means to identify the toxin in cells, as well as to measure its removal rate from blood. By combining fluorescent labeling as a tracer for the toxin with fluorescent antibody methods, we have been able to correlate the vascular clearance of the toxin with its cellular localization.

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A version of this paper will be published elsewhere.

Staphylococcal enterotoxemia was induced in rats and monkeys by IV injection of purified SEB. The biologic distribution of the unlabeled toxin was then assessed by fluorescent antibody methods¹¹ 1 hr later. No significant differences in distribution were observed at this time period between the 2 animal species for the tissues examined (Table I). In both species, a minimal positive reaction was observed in spleen and gastrointestinal tract whereas the lung and multiple sections at various levels of the brain including cortex, pons, and medulla were negative. The liver of the monkeys had occasional but infrequent fluorescence whereas the liver of the rat failed to show significant localization. In contrast to these organs, the kidneys of both species exhibited a brilliant green luminescence. The location of this fluorescence appeared to be confined to the proximal convoluted tubules as little fluorescence was observed in distal tubules; the glomeruli were consistently negative.

Table II summarizes the data now available on the vascular clearance of SEB. The results were tabulated from observations made on 4 or more animals. The data cited for SEB-I¹³¹ in monkeys is from Rapoport et al.⁸

Although labeling of the toxin with either a fluorochrome or radioactive iodine did not destroy its toxicity, the labels did have an effect on the rate of clearance of the toxin from the circulation. Thus, when SEB labeled with radioactive iodine was injected into rats, its clearance was significantly faster than the clearance constant for SEB labeled with fluorescein isothiocyanate (FIT).

However, following bilateral nephrectomy, there was a dramatic retardation in the clearance of SEB regardless of the label. Since the rate of vascular clearance was actually composed of the sum of the clearance rates of the different organs, this reduction in clearance rate could be used to estimate the clearance rate constant of the kidney; that is, the difference in rate constants before and after nephrectomy. The percentage of the challenge dose cleared by the kidney was estimated by substituting this constant into the integrated form of the differential equation that expressed kidney removal in terms of the blood toxin concentration. By this method, 60 min after injection between 75 and 80% of the toxin regardless of label and in both rats and monkeys theoretically should have been cleared by the kidney. This observation underscored the importance of the kidney in the removal of IV injected toxin and confirmed the impression of the distribution of SEB as indicated by fluorescence microscopy and reported in Table I.

The question next arose as to the mechanism of renal clearance. Therefore, the following sets of experiments were performed in order to determine whether the SEB localized in the renal tubules by a process of glomerular filtration and tubular reabsorption or by pinocytosis from the efferent glomerular blood.

TABLE I. BIOLOGIC DISTRIBUTION OF SEB ONE HOUR AFTER CHALLENGE

SEB LABEL		ANIMAL		METHOD		DISTRIBUTION BY TISSUES				
				Kidney	Liver	Lung	Spleen	Gastrointestinal tract	Brain	
None	Rat	Fluorescent antibody		+++	±	-	±	±	--	
FIT	Rat	Fluorescent microscopy		+++	+	-	±	±	-	
I125	Rat	% Injected dose		56.7	5.4	0.68	1.22	4.49	0.03	
None	Monkey	Fluorescent antibody		+++	±	-	±	±	-	
FIT	Monkey	Fluorescent microscopy		+++	±	-	±	±	-	
I-131	Monkey	% Injected dose		33.0	19.5	5.0	0.15	6.0	0.2	

TABLE II. CLEARANCE DATA AND TOXIN DISTRIBUTION IN KIDNEY

SEB LABEL	ANIMAL	BLOOD CLEARANCE CONSTANT, K		KIDNEY CLEARANCE CONSTANT, K	THEORETICAL % INJECTED DOSE IN KIDNEY
		Control	Post- nephrectomy		
FIT	Rat	0.082	0.015	0.067	81.1
I ¹²⁵	Rat	0.121	0.031	0.090	74.3
I ¹³¹	Monkey	0.096 ^{8/}	0.022 ^{8/}	0.074	76.8

The objective of the first set of experiments was to determine the earliest localization of the toxin in the renal tubules and to correlate its subsequent distribution with the time after challenge. It was reasoned that if the kidney samples were taken at sufficiently close intervals after challenge it might be possible to observe (a) whether the toxin was initially present within the tubular lumen and (b) the direction of migration of the toxin across the cell. The experiments were done with both unlabeled SEB detected by fluorescent antibody methods and with SEB conjugated with FIT. Both techniques gave essentially similar results.

The earliest time postchallenge at which the toxin could be visualized in the kidney tubules was 15 sec; the toxin was clearly present on the luminal side of the proximal convoluted tubules as a distinct thin layer of fluorescence adjacent to the cell surface. In some sections, it was possible to observe that the toxin was present at the glomerular-tubular junction. However, it was confined to the tubular epithelium and was not observed adherent to the parietal layer of Bowman's capsule or to glomerular capillary endothelium. Indeed, the toxin appeared to show an affinity only for the brush border of tubular epithelium. From 15 sec to 1 min, the intensity of the fluorescence increased but its distribution relative to the cell did not change. From Figure 1A it is clear that the toxin is still confined to the tubular lumen.

Five minutes after injection, the toxin was still located predominantly on the luminal surface of the tubular cell. However, by 15 min the toxin began to be seen within the cell and there was a definite gradient of fluorescent intensity from the luminal side of the cell towards the base of the cell. By 30 min the entire tubular cell was fluorescent (Figure 1B). In all sections examined the presence of fluorescence was confined to the proximal tubules and did not involve the glomerulus or the distal segments.

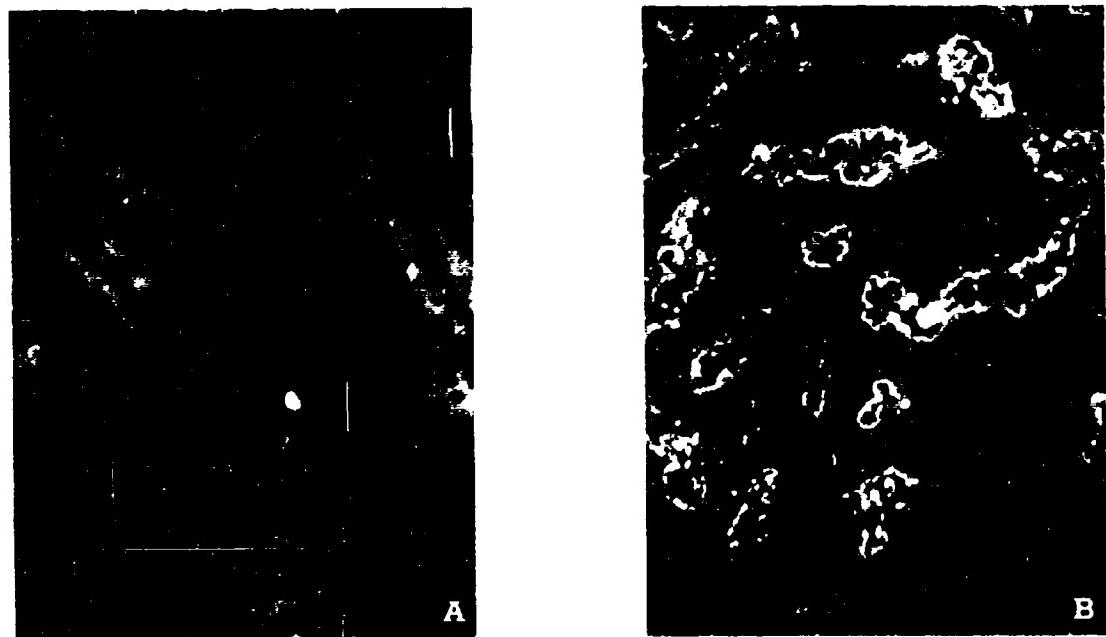


FIGURE I. LOCALIZATION OF SEB IN RAT KIDNEY AFTER INJECTION. (FLUORESCENT ANTIBODY METHOD, 820 X). A. AT 1 MIN, FLUORESCENCE ON LUMINAL BORDER. B. AT 30 MIN, ENTIRE CELL INVOLVED).

It was concluded that the earliest appearance of either unlabeled SEB or SEB-FIT was on the luminal side of the tubular cell presumably adherent to the brush border of the proximal convoluted tubule. The SEB then migrated from the luminal side to the basal portions of the cells so that by 30 min the entire cell was fluorescent.

These findings strongly indicated that SEB was filtered by the glomerulus and reabsorbed by the proximal tubules. If this were so, then it would be possible to prevent the localization of SEB in the kidney by a change in tubular pressure. Such a change can be induced by ureteral ligation. For this experiment, a unilateral ligation was performed so that the opposite kidney could serve as a control. SEB-FIT was then administered 3, 6, 12, 24, 48 and 72 hr after ligation and tissue was obtained 30 min later. Sections of kidney were examined for the presence of fluorescence and with light microscopy for any attendant pathological changes.

In kidneys obtained at 3 and 6 hr, the ligated and control kidneys showed an equal intensity of fluorescence. However, by 12 hr there was a marked reduction in fluorescent intensity in those kidneys whose ureters had been ligated. This change was more pronounced at 24 hr but failed to show any further appreciable alterations in sections obtained at 48 and 72 hr. Figure 2 shows control and 24-hr sections. Even as late as 72 hr, some tubules surrounding single glomeruli continued to show fluorescence although most of the renal cortex was devoid of identifiable SEB. No appreciable pathologic changes were evident in sections obtained prior to 48 hr after ureteral ligation. It was concluded that a change in tubular hydrostatic pressure could prevent the localization of SEB in the kidneys.

If SEB were filtered by the glomerulus, then some relationship should exist between the rate of clearance of SEB and the rate of glomerular filtration. The calculation of glomerular filtration rate (GFR) could be made providing it was assumed that all SEB reaching the glomerulus was filtered and that SEB was not removed by the kidneys in any other manner. Under these conditions GFR was equal to the product of the clearance rate constant of the kidney and the animal's blood volume. For a 100-gm rat with blood volume estimated at 6.2 ml,^{12/} this yielded a figure for SEB-FIT of 0.42 ml/min and 0.56 for SEB-I¹²⁵ (Table III). According to Smith,^{13/} the best figure for rat GFR is 0.60 ml/min per 100 gm body weight. Similarly for the monkey, based on SEB-I¹³¹ and a blood volume of 44.7 ml/kg,^{14/} the GFR was 3.31 ml/min per kg as compared to 3.66 ml/min per kg recorded by Morris and co-workers using creatine clearance.^{10/} The remarkable similarity between the accepted value for GFR and that derived from SEB clearance was consistent with the hypothesis of SEB filtration.

The low molecular weight of the toxin (35,300)^{3/} would be consistent with glomerular filtration. However, Crawley and co-workers,^{5/} who investigated the blood binding properties of SEB precipitation methods reported that 90% of IV injected SEB was transported in blood as a complex

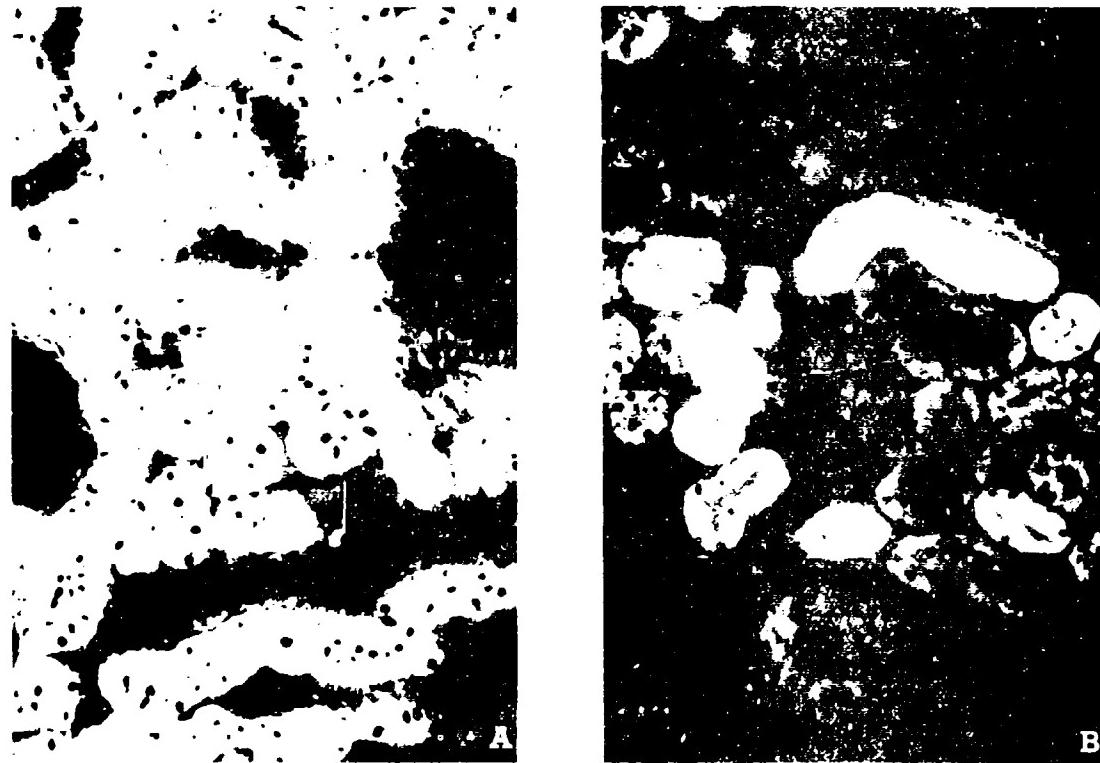


FIGURE 2. LOCALIZATION OF SEB IN RAT KIDNEY 30 MIN
AFTER INJECTION. (FLUORESCENT ANTIBODY
METHOD). A. CONTROL (250X). B. URETERAL
LIGATION 24 HR EARLIER (820X).

TABLE III. ESTIMATION OF GLOMERULAR FILTRATION RATE

TOXIN	ANIMAL	VOLUME BLOOD	GFR ml/min	
			SEB	ACCEPTED
SEB-FIT	Rat	6.2 ml/100 gm	0.42	0.60
SEB-I ¹²⁵	Rat	6.2 ml/100 gm	0.56	0.60
SEB-I ¹³¹	Monkey	44.7 ml/kg	3.31	3.66

with serum albumin. From the study presented here, it now appears to be firmly established the IV administered SEB is rapidly removed from the circulation with concomitant accumulation in proximal convoluted tubules. Since it would be difficult to equate rapid vascular clearance with glomerular filtration, if indeed the toxin was bound to a circulatory transport protein, the next set of experiments was initiated to reexamine by chromatographic, electrophoretic and immunologic methods the vital question of whether SEB exhibited any affinity for blood protein.

Since an affinity between SEB and albumin had been reported, preliminary experiments were performed using purified bovine serum albumin (BSA) and SEB. It was reasoned that, if a complex were formed between SEB and albumin, the electrophoretic character of the complex should be different than that of either SEB or albumin. When 1 mg/ml of albumin was mixed with 1 mg of SEB at 37 C for 30 min and electrophoresis was performed, no such intermediary was found (Figure 3, SEB + BSA). Although no apparent binding had occurred between SEB and albumin, the experiments were extended to include monkey plasma for the possibility existed that difference in binding might arise due to species differences or to the albumin preparation. However, when 1 mg of SEB was added to 1 ml of monkey plasma at 37 C for 30 min, there was no apparent change in the electrophoretic behavior of the SEB due to plasma addition (Figure 3, SEB + plasma). Thus, SEB with its isoelectric point of 8.6 traveled towards the cathode in agar gel of pH 7.4 and appeared slightly ahead of the γ -globulins. Despite this appearance of SEB at a zone corresponding to SEB alone, it did not exclude some SEB from binding to serum proteins and particularly to the γ -globulins. Therefore, analysis by immunolectrophoresis was performed in order to enhance visualization of the location of SEB.

Immunolectrophoresis was performed using both rat and monkey plasma with similar results. A typical pattern from the rat is presented in Figure 4. Electrophoresis of SEB and plasma (A) was first performed and compared to the location of an equal concentrate of SEB in saline. In both instances, only a single precipitin band to rabbit anti-SEB was found; it was apparent that plasma had not altered the mobility or identified the

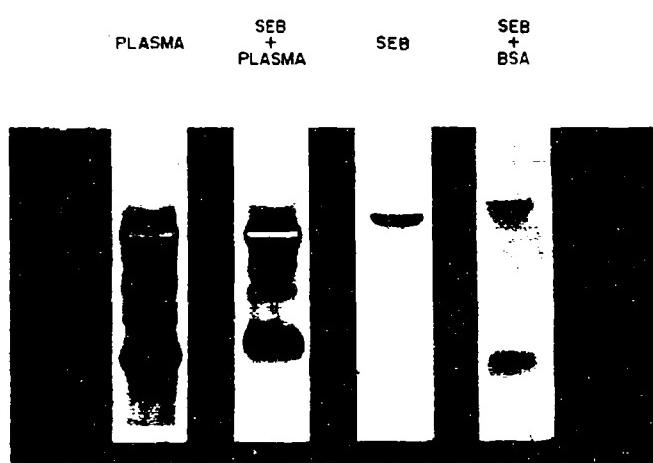


FIGURE 3. ELECTROPHORESIS OF SEB UNDER VARIOUS CONDITIONS.

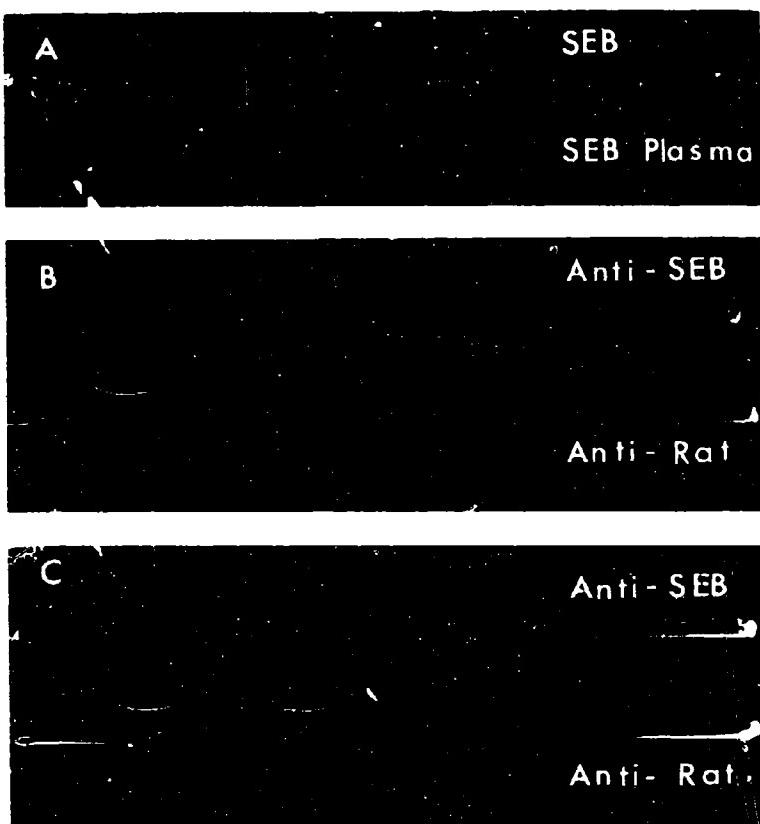


FIGURE 4. IMMUNOELECTROPHORESIS OF SEB UNDER VARIOUS CONDITIONS.

fraction of SEB. In order to observe whether the location of the SEB coincided with a plasma protein component, the plasma containing SEB following electrophoresis was allowed to diffuse in one direction against rabbit anti-rat whole plasma and in the other direction against rabbit anti-SEB. (B) is the pattern obtained when the SEB was added to plasma in vitro while (C) was obtained with plasma 3 min after injection of SEB in vivo. As seen here, there was no identity between the SEB band and any of the identifiable components of plasma. A similar result was obtained regardless of whether the SEB was mixed with plasma in vitro or added to plasma by IV challenge.

Analysis of Ouchterlony gel diffusion studies also failed to reveal any evidence of association between SEB and plasma proteins. A typical experiment using monkey plasma is presented in Figure 5. The center well contains rabbit antiSEB. Only a single precipitin line is seen between plasma SEB and rabbit antiSEB and it is continuous with the precipitin line formed with SEB in saline. The fact that the precipitin line appeared at the same distance between SEB in saline and antiSEB as between SEB in plasma and antiSEB, when equal concentration of SEB were used, indicated that the coefficient of diffusion of SEB alone and SEB plus plasma was identical. Furthermore, when the plasma components were identified with goat antimonkey, it was apparent that the SEB precipitin line extended completely across the lines of all major plasma components without evidence of discontinuity, identity or partial identity with any major plasma component.

The electrophoretic and immunologic evidence now available indicating an absence of association between SEB and plasma proteins was supported by experiments using column chromatography (Figure 6). Since the molecular weight of SEB at 35,300 is less than most plasma proteins, it was possible to separate SEB from plasma using molecular sieve chromatography. A column of G-75 Sephadex was selected in order to exclude from the gel bed nearly all blood proteins including albumin while retarding the migration of SEB. The toxin was identified by a fluorescent label and the protein by the Biuret procedure.¹⁵⁷ When SEB alone was applied to the column, it was eluted in a volume significantly greater than the protein peak evident when plasma alone was applied. Therefore, it would be expected that when plasma and SEB were present together and if SEB were bound to a blood protein, it should migrate at a faster rate and appear in the plasma protein peak. However, when such an experiment was performed and the SEB was added to plasma either by injection in vivo or by incubation in vitro, there was no significant increase in the fluorescence of the plasma protein peak. Similar results were obtained with both rat and monkey plasma.

Although several different procedures were used in attempting to establish an association between SEB and blood proteins, no complex could be identified. Whereas a failure to identify a complex by immunologic methods might occur if the immunologic character of the SEB had been altered by complex formation, this possibility was excluded by the chromatographic experiments in which the elution to void-volume ratio of SEB was not altered by plasma addition and by the fact that on serial dilution all of the SEB in

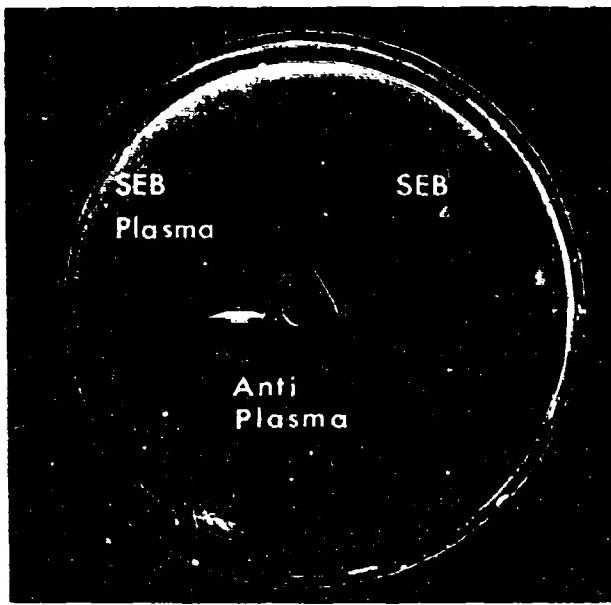


FIGURE 5. OUCHTERLONY GEL DIFFUSION OF SEB AGAINST
SEB + PLASMA AND ANTIPLASMA.

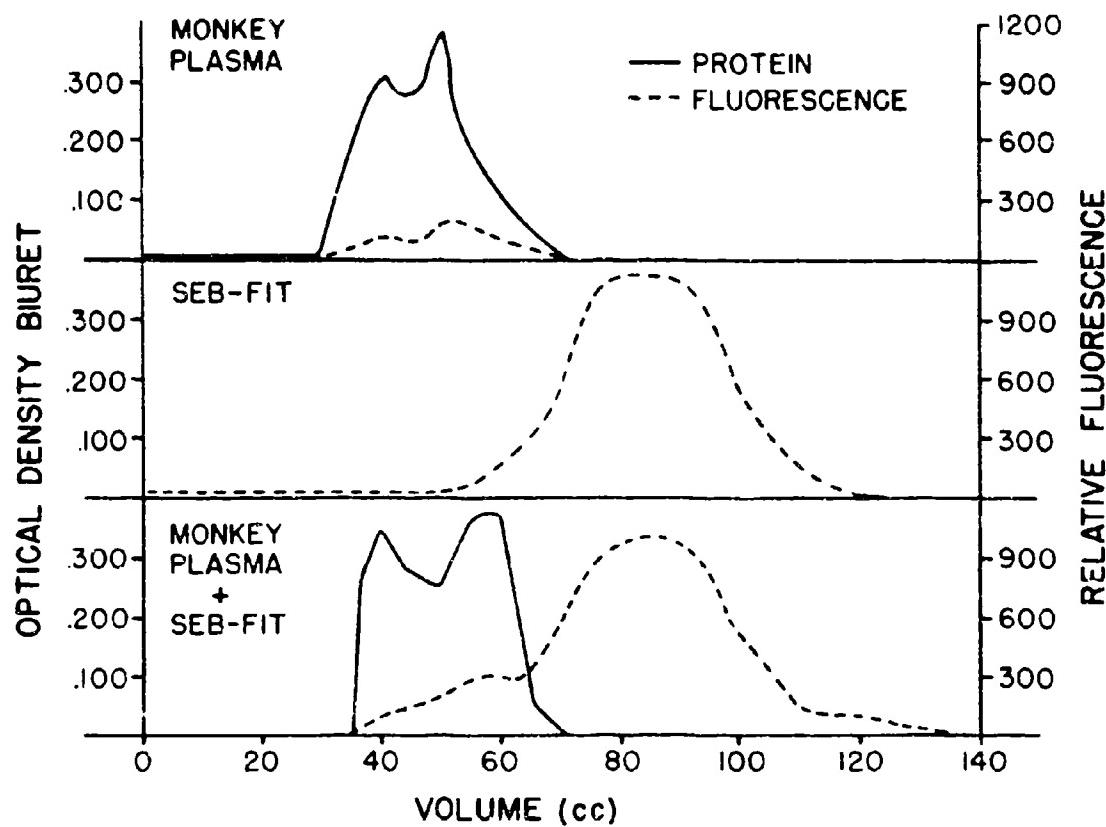


FIGURE 6. CHROMATOGRAPHIC SEPARATION OF SEB FROM PLASMA PROTEINS.

plasma could be accounted for as a single band on immunoelectrophoresis. The present studies, therefore, demonstrate the unlikelihood of association between SEB and plasma proteins and imply the restriction that if such binding does occur it must be of very weak character and easily dissociable.

It is concluded that SEB when present in the circulation of a nonimmune animal is neither transported by nor associated with any major protein of the blood. This conclusion implies that the behavior of the toxin in the circulation and its biologic fate following IV inoculation probably reflect the intrinsic properties of the SEB molecule itself. Therefore, it appears likely that the rapid removal of SEB from the circulation following IV challenge is a manifestation of its low molecular weight. This property by predisposing the toxin to efficient glomerular filtration appears to account for the vascular clearance and contributes to the early and rapid renal accumulation of toxin.

SUMMARY

Experimental enterotoxemia was induced in rats and monkeys by IV injection of purified SEB. The clearance of the toxin from the blood and its biologic distribution were examined. Differences in clearance rate were found depending on the means by which the toxin was identified. However, the toxin was rapidly removed from the circulation principally by means of renal clearance. This conclusion was supported by distribution studies and by the dramatic drop in clearance rate as a result of nephrectomy. Distribution of the toxin was studied in relation to its concentration in whole organs by means of radioactivity and to its cellular localization by means of fluorescence microscopy. Fluorescent labeled and unlabeled toxin identified by fluorescent antibody methods was consistently seen only in renal proximal convoluted tubules, although low amounts of radioactivity were detected in liver and gastrointestinal tissue. It was proposed that toxin gained access to these tubules by a process of glomerular filtration and tubular reabsorption because (a) toxin was first seen on the brush border of the tubules and was subsequently seen to migrate across the cells, (b) changes in tubular hydrostatic pressure prevented renal accumulation of toxin, (c) the rate of clearance by the kidney was equivalent to the rate of glomerular filtration, and (d) the low molecular weight of the toxin (35,300) in the absence of demonstrable association between SEB and blood protein would predispose the toxin to efficient glomerular filtration.

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DISCUSSION

DR. GREISMAN: I'd like very much to know about this renal clearance and uptake by the kidney: is this a protective mechanism or is this the harmful mechanism to the host?

DR. NORMANN: There is some evidence to suggest that the toxin which is filtered by the glomerulus does not leave the kidney by way of the urine and that nearly all the SEB is reabsorbed by the tubule. In this sense one might believe that the process is not a protective one because the toxin remains in the organism and is not removed. In addition, there is the question of whether the toxin in the kidney is the significant localization or whether the toxin may act at sites other than the kidney. We are now concerned with experiments over a longer time period, -- that is, in a period greater than an hour -- and these experiments indicate that the concentration in monkey kidney is more or less transient, lasting about 4 hr. This observation in turn raises the question where does the toxin go after it leaves the kidney? Returning to your original question, there is some evidence that the kidney may not be the site of action of the toxin. For example, there is no alteration in the functional status of the kidney prior to overt shock; there is no change in the kidney morphologically by electron microscopy; and we can not demonstrate an alteration in certain enzymes which we have looked at histochemically, so I think these three things might suggest that the toxin does not injure the renal cells directly.

DR. CLUFF: If you were to ligate the renal vessels and inject the toxin is death shortened or prolonged?

DR. NORMANN: Death occurs and I believe Dr. Beisel has additional information since he has been concerned with this experiment.

DR. CLUFF: Do ligated animals die more quickly or less quickly Dr. Beisel?

DR. BEISEL: They appear to die about the same time. There is an old experiment in the literature that Dr. Staab attempted to reproduce; Israel originally used crude staphylococcal broth filtrates. If he gave toxin in broth to rabbits, waited a brief period of time, and nephrectomized the animals, he could get an increased short-term survival over nonnephrectomized control rabbits given crude toxin. Perhaps this was merely an artificial means of lowering the dose if the toxin was primarily localized in the kidney. By removing the kidney and the toxin localized within it, he may have in effect, lowered the dose at some other site of action. We

do not know if the kidney transforms the toxin into a more lethal molecule, or if it merely removes toxin from the blood as the kidney does with other protein molecules such as insulin or albumin.

DR. CLUFF: This looks like tubular reabsorption, doesn't it?

DR. HIRSCH: When we talked about this last year I recalled that there was a very striking delay in action of enterotoxin, about an hour and a half? You don't get prompt toxicity even if you give purified toxin IV. This very interesting delay suggested strongly that something might happen to the injected toxin molecule before it becomes the "true" toxin. This was presented a year or so ago and there was demonstrated clear localization in the kidney. This brings up the intriguing possibility that during tubular reabsorption something happens to this molecule. The process of tubular reabsorption, for this type of protein molecule, probably represents pynocytosis, as has been studied with some other protein molecules. In a pynocytic vacuole, digestion, alteration, or other modification of the molecule could very well occur. It seems entirely reasonable that what emerges from the tubular cell may be the really toxic thing, which then becomes fixed to the vessels or other sites to cause real trouble. So I'd be very interested if you have done experiments in which the toxin is given to nephrectomized animals or animals that have had their renal artery ligated to be sure that this sort of thing isn't happening.

DR. BEISEL: This has been attempted by Dr. Staab, working primarily with monkeys, but the number of animals needed is going to be very large to find any statistically significant differences.

DR. HIRSCH: The differences aren't that great?

DR. BEISEL: That's right.

DR. HIRSCH: I suppose that both molecules might be toxic: in other words, there would be multiple toxic effects from both the unmodified and the modified molecule.

DR. CLUFF: Why would not simple storage account for the delay, without requiring a change in the chemistry of the toxin. Perhaps by its early localization in the kidney, it isn't poisoning the heart, the brain or the gastrointestinal tract: then there is a slow release, a delayed form of administration of toxin.

DR. HIRSCH: Well if that same unchanged toxin is fixed at the eventual sites of toxicity in the central nervous system or elsewhere, you'd think that the big dose you give IV the first time would be immediately affixed to those sites.

DR. CLUFF: Unless there is some circulatory bypass, I agree with your point. I don't know.

DR. BEISEL: It may take a toxin with a built-in tritium tag to obtain this information. If parts of the molecule become dissociated, we could still follow the residuals. The toxin may have an active "core" as some hormones do.

DISCUSSION OF SECTION VI

Elisha Atkins, M.D.*

All the very shrewd questions on the tip of my tongue have been asked by other members on the panel so I can fix my speculations more narrowly toward Dr. Greisman's elegant work. This is in an area that I have worked in exclusively. In fact, as someone who has never written or talked about anything professionally except the pathogenesis of fever, I can appreciate the thrust of George Bernard Shaw's comment when he said, "A specialist is somebody that knows everything about his own subject, except his relative unimportance."

One question before us involves the definition of a toxin. In the two latter papers we have seen some beautiful examples of work almost certainly dealing with a highly purified material; so one doesn't have to worry about inadvertent contamination with endotoxin. As is widely recognized, such a contamination can cause a great deal of grief in animal studies, or even when microbial agents are introduced into tissue cultures. No matter how sophisticated the experimental design, if the material one introduces isn't what you think, you're going to arrive at the wrong answers. And the problem of toxin contamination is a very real one. I see it all the time because I am asked to perform tests on the possible pyrogenicity of materials introduced into patients during catheterization or other studies of a similar nature. As an example we tested some very "pure" streptolysin S given us by a colleague; this was so grossly contaminated with endotoxin that I hate to think what conclusions to draw from the very elegant studies on pathogenesis of arthritis conducted with the same material. So this is a very real question in terms of trying to assess experimental results of a variety of studies.

One of the very interesting things to those of us who still look at patients is that, with the exception of Gram-positive cocci that produce diseases with high and prolonged fevers, the Gram-positive bacilli causing such diseases as tetanus, botulism, staphylococcal enterotoxemias and Clostridial diseases cause illness with much less fever than those seen with the common infections of the genitourinary tract due to Gram-negative organisms. In terms of correlation, one gets soluble products in the test tube when one cultures Gram-positive organisms whereas Gram-negative bacilli all elaborate a potent endotoxin characterized by both fever and tolerance-producing abilities which have been shown so nicely by Dr. Greisman's study. His study brings up some very interesting problems about both the pathogenesis of fever and its opposite, the pathogenesis of tolerance. We're used to thinking of antigen-antibody complexes as toxic; study of the effects of such complexes in the kidney demonstrates this very clearly.

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Yet here we have a situation where the animal actually is getting less reactive while developing antibodies that presumably are blocking the toxic part of the molecule. One presumably should have circulating antigen antibody complexes so it is hard to see why the animal doesn't continue to have fever as it does when given any of a great variety of particulate materials. If you wash Gram-positive organisms and inject them you can get reproducible fever. One can liberate pyrogen when leukocytes of the host are incubated with these organisms.

The whole question of antibodies which block toxicity has to be balanced against certain antibodies that obviously promote toxicity. In this instance we have animals that are sensitized through infection, e.g., with BCG. These animals become sensitive to the toxic action of tuberculin interestingly enough; although this is always taken as a model of delayed hypersensitivity, one can transfer a fever with the serum of an animal that was made hypersensitive by infection. Give its serum to a normal animal, challenge that animal for the first time with tuberculin, and one gets reducible tuberculin fever. Now Farr and his group have shown a similar phenomenon with nonmicrobial antigens, using bovine serum albumin. One can do it also by sensitizing animals with repeated injections of human serum albumin (HSA). Transfer the serum of that animal into another animal; challenge that animal with HSA and one produces fever. So here is a case where the antibody is actually acting in conjunction with the antigen to cause the cellular damage which ultimately leads to the release of pyrogen and fever. Whereas in the development of tolerance, one presumably has another sort of antibody intervening and protecting the tissue in some way from liberating pyrogen and producing fever.

Now we have attacked this problem by incubating endotoxin and a variety of microbial and other antigens with various tissues, usually those that contain leukocytes because leukocytes seem to be the one source of pyrogen production. The leukocytes produce pyrogen; they seem to require energy and protein synthesis to do so. One can block this by putting in such things as puromycin, but when one adds these stimuli to leukocytes of animals, either granulocytes or monocytes, one can activate these cells so that they in turn will release the pyrogen in vitro. Now recently we have taken the liver and separated it (by a very simple technique involving EDTA) into Kupffer and hepatic parenchymal cells. When we add any one of a variety of microbial activators (e.g., endotoxin or tuberculin) to cells of sensitive animals, we can liberate pyrogen only from the Kupffer cells but not from the parenchymal cells. The interesting thing is, that when we take these livers from animals that have developed tolerance of the 1-day variety, that Dr. Greisman calls "desensitized" (and I share this hypothesis for this early tolerance) we find that the Kupffer cells fail to release any pyrogen.

So again, there may be some aspect of tolerance that has to do with the target cell, and it makes no difference whether this cell is in normal serum or in the serum of a tolerant animal. It is not the serum of a

tolerant animal which protects that cell in any way from releasing pyrogen when one adds an antigen or toxin to it in vitro. These are some of the complexities of this work. The thing that particularly appeals to me is that our last three speakers, starting out with well defined illnesses and very elegant techniques, have thrown a great deal of light on probable mechanisms of disease as we see them clinically.

CONCLUDING REMARKS

Colin M. MacLeod, M.D.*

DR. T. E. WOODWARD: We have all been rewarded by attending this 2-day session and certainly we are extremely grateful to those who made the presentations, to the moderators and to the discussants. It would seem most appropriate at this time, to call upon that person among us who is most qualified to recall this material and present us a summary of the proceedings, Dr. MacLeod.

DR. MacLEOD: Well, Dr. Woodward, I'm afraid that I'm not going to be able to fulfill that very large task. There has been a very wide range of subjects covered in these meetings, beyond anyone's capacity to really prepare a summary. However, congratulations are in order to all of those participating in the organization of this excellent symposium. On behalf of the Commission, I would like to thank all of the participants.

There have been many good and new observations made during the course of this 2-day meeting, including Dr. Levine's somewhat complicated diagnostic method for mycoplasmal infections of tissue culture cells. One of the things most pleasing to me is the remarkable progress that the Medical Unit itself has made in developing the comprehensive approach to the study of the metabolic alterations that take place during infection. I congratulate them on the progress they have made. Now, as is apparent from our meeting, a host of metabolic changes take place during infection. What remain the largest areas of darkness are the mechanisms that underly such changes, as well as the significance of the changes themselves in the infectious process and in defenses by the host against the damage caused by infection.

I suppose the changes can be classified in a number of ways as Dr. Ingbar did very successfully in his discussion this morning. One might say the changes could be classified in somewhat the following way: first, as to whether the observed changes are direct responses of cells in tissues to the infectious agents. As examples of this, consider the destruction of cells because microbes are growing within them; damage to the cells by the toxic products of the microbe; or damage suffered by phagocytic cells in the course of the scavenger process. These would be direct effects.

The second set of changes would include those that are associated with the stress reactions, i.e., changes that effect the hypothalamic, pituitary, and endocrine systems and lead to alterations that are distinct from those due to direct interaction of the microbe in the host tissue.

* The Commonwealth Fund, New York, New York.

The third set would be those deriving from the microbe itself, i.e., new enzymes appearing as a consequence of virus multiplication during virus infection, or the products of infectious agents such as extra-cellular protein or perhaps capsular polysaccharides or related products.

The fourth set of changes of course are those associated with the immunological response, the production of the immunoglobulins for example. Fifth, there are those effects described particularly by Dr. Gray that are associated with fever itself.

A sixth group might be the products that are not clearly associated with any of the others, such as the emergence of C-reactive protein which was not discussed in the meeting, or possibly the increased glycoproteins.

A seventh category would be a mixture of all of these, which is probably what is usually going on in the course of an infectious disease.

It is well to recall that many of these effects are nonspecific, in that they are seen in the course of infection by a wide variety of very different agents, or following cell or tissue damage caused by simple chemical agents, by physical agents or by trauma. We cannot be sure that any of these nonspecific changes may have much bearing on resistance to infection, or to the damage caused by infection. If one could prevent them or reverse them in the infected host it is not known if the outcome would be different than if they were permitted to proceed without intervention. This may seem like a very strong statement but the issue needs to be presented in such stark terms. I am not, of course, attempting to, and do not wish to, down-grade the possible significance of the metabolic changes that have been observed. I am pleading rather for a hard look at their significance. It would seem to me desirable, for example, to devise experimental situations in which, insofar as possible, the various kinds of change could be reproduced in a relatively pure form, that is, in a form uncomplicated by the problems introduced by multiplying microbes in the cells and in the tissues. I am not prepared really to outline how this can be done. For example, immunological reactions can be avoided in one's analysis through the use of nonantigenic agents. Changes caused by phagocytosis can perhaps be isolated; I leave this to Dr. Hirsch.

The role of the adrenal has been quite extensively studied so that we know a good deal about its participation, but of course not nearly enough in relationship to other concomitant changes. Now, if one can devise means to separate out, insofar as possible, the adrenal changes, we could begin to undertake more effectively the study of the mechanisms which produce the changes, and so learn the significance of them. In saying this, I am of course aware that good attempts have been made to do this and to analyze the nature of some of the changes, particularly those related to stress reactions.

One observation seems pertinent in the case of enzymes found free in the blood or in organ extracts. These presumably arise from cells which have been damaged so that they become "leaky," their integrity is actually broken, or they are lysed. Does the toxicity experienced in diseases caused by agents that do not have exotoxic products result from the products released from the host's own cells? Various enzymes or metabolic intermediates, for example, that cause secondary and progressive damage to other cells? Is this what happens in extensive burns which often proceed so inexorably to fatal outcome? I am interested that no attention was given to C-reactive protein. That was an old love of mine that I had pretty much forgotten about. I don't think that it necessarily deserves special attention, but it is another example of a nonspecific reaction to tissue and cell damage. But once again, as in the other reactions to cell injury, we know little about its origin; we know little about its mechanism of release, nor do we know its role in tissue damage if it has any. We need to direct our attention toward the mechanism and the significance of these reactions and changes that occur as consequences of infection.

I've been very pleased to have the opportunity to be present during the last two days. The comprehensive nature of this Symposium and the good discussion that has taken place will cause a lot of us to have new, and perhaps different or hopefully better, ideas about the experimental procedures that we need to employ in the future.

DR. T. E. WOODWARD: It was my privilege to hear Dr. MacLeod summarize a conference on cholera in Honolulu several years ago and I can assure you that it was equally succinct.

PUBLICATIONS OF THE U. S. ARMY MEDICAL UNIT

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